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UNITED STATES DISTRICT COURT
for the
DISTRICT OF NEW JERSEY

CHAYA GROSSBAUM and MENACHEM GROSSBAUM, her spouse, individually and as *guardians ad litem* of the infant ROSIE GROSSBAUM,	Hon. Garrett E. Brown, Jr.
Plaintiffs,	CIVIL ACTION NO. 07-CV-1359 (GEB)
vs.	
GENESIS GENETICS INSTITUTE, LLC, of the State of Michigan, MARK R. HUGHES, NEW YORK UNIVERSITY SCHOOL OF MEDICINE and NEW YORK UNIVERSITY HOSPITALS CENTER, both corporations in the State of NEW YORK, ABC CORPS. 1-10, JOHN DOES 1-10,	**CERTIFICATION OF COUNSEL RE: DECLARATION OF EXHIBITS**
Defendants.	

I, Lewis Stein, Esq., certify that:

1. I am a partner in the law firm of Nusbaum, Stein, Goldstein, Bronstein & Kron, P.A., a member in good standing of the bar of this Court, and counsel of record for the Plaintiffs, Chaya Grossbaum, Menachem Grossbaum and their child, Rosie Grossbaum. In this capacity, I am familiar with the facts of this matter.

2. This Certification is being submitted in support of Plaintiffs' opposition to Defendants' Motions for Summary Judgment and to bar expert testimony.

3. The exhibits cited in and submitted with the Supplemental Statement of Undisputed Material Facts and in support of Plaintiffs' opposition to Defendants' Motions for Summary Judgment and to bar expert testimony are true and correct copies which are relied upon by the Plaintiffs in support of the opposition.

These exhibits are as follows:

1 Excerpts from An Atlas of Preimplantation Genetic Diagnosis edited by Yury Verhinsky, Ph.D., Anver Kulier, M.D., Ph.D. of Reproductive Genetics Institute, Chicago, IL (2000).

2 Introduction to Genetics from Wikipedia, the free encyclopedia, wikipedia.org/wiki/Introduction_to_genetics.

3 Declaration of Plaintiff, Chaya Grossbaum.

4 "Review: Molecular Diagnostics in Preimplantation Genetic Diagnosis" by Thornill, AR and Snow, K, Journal of Molecular Diagnostics, Vol. 4, No. 1, pp. 11-29; February 2002.

5 "Multiplex PCR of polymorphic markers flanking the CFTR gene; a general approach for preimplantation genetic diagnosis of cystic fibrosis" by Dreesen, JC, et al, Molecular Human Reproduction, Vol. 6, No. 5, pp. 391-396, 2000.

6 Excerpts from deposition transcript of Mark Hughes, M.D. dated 2/19/09.

7 Excerpts from deposition transcript of Charles Strom, M.D., Ph.D. dated 5/4/10.

8 Genesis Genetics record- DNA Sequencing - Genotyping Assay dated 7/18/04.

9 Genesis Genetics record- "Message" dated 7/19/04.

10 Genesis Genetics record- three page "Final Report" dated 7/19/04.

11 Excerpts from deposition of Dr. Mark Hughes dated 5/14/10.

12 *Curriculum vitae* of Charles Strom, M.D., Ph.D.

13 Genesis Genetics- page from website www.genesisgenetics.org.

14 Defendants, Genesis Genetics' and Mark Hughes, certified Answers to Interrogatories.

15 Resumé of qualifications of Defendant, Mark Hughes, M.D. provided with report of 3/2/10.

16 Excerpts from deposition of Samuel Pang, M.D. dated 11/23/10.

17 Marine Biological Laboratory Lecture Series, abstract of lecture and resume of Defendant, Mark Hughes, M.D. dated 7/16/04 at www.mbl.edu.

18 Excerpts from deposition transcript of Kangpu Xu, M.D. dated 5/13/10.

19 Excerpts from deposition transcript of Charles Strom, M.D. dated 6/24/10.

20 Report of Kangpu Xu, M.D. dated 2/26/10.

21 Report of Mark Hughes, M.D. dated 3/2/10.

22 Excerpts from deposition of Frederick Licciardi, MD dated 3/11/09.

23 Excerpts from deposition of James Grifo, M.D. dated 6/24/09.

I certify that the foregoing statements made by me are true. I am aware that if any of the foregoing statements made by me are willfully false, I am subject to punishment.

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Date: February 15, 2011

PART I

PLAINTIFFS' EXHIBITS

EXHIBIT 1

THE ENCYCLOPEDIA OF VISUAL MEDICINE SERIES

An Atlas of PREIMPLANTATION GENETIC DIAGNOSIS

Yury Verlinsky, PhD
and
Anver Kuliev, MD, PhD
Reproductive Genetics Institute
Chicago, Illinois

With a foreword by
Robert G. Edwards
Cambridge, UK

The Parthenon Publishing Group
International Publishers in Medicine, Science & Technology
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Section I Review of Methods and Experience in Preimplantation Genetic Diagnosis

Preimplantation genetic diagnosis (PGD) is a principally new approach for the prevention of genetic disorders, which avoids the need for prenatal diagnosis and termination of pregnancy. It is based on control of the processes of oocyte maturation, fertilization and implantation, to select and transfer back to the uterus only normal embryos, and achieve an unaffected pregnancy and the birth of a healthy baby. In this way, couples at high risk of having offspring with genetic disease will have an option to control the outcome of their pregnancy from the very outset. Although this option involves ovarian hyperstimulation and *in vitro* fertilization (IVF), available experience shows that PGD appears to be an acceptable procedure in many ethnic groups all over the world.

The application of PGD is of particular interest for assisted reproduction practices, because genetic factors contribute considerably to infertility problems. Moreover, the introduction of the methods for preselection of euploid embryos for transfer may considerably improve the efficiency of IVF, because aneuploidies are one of the major contributors to spontaneous abortions and may also explain some implantation failures. Although at present there are not enough data to confirm this, relevant data are currently being collected and will be available in the very near future.

PGD may be achieved by testing female gamètes or single cells from the preimplantation embryo. Both of these methods have been used in clinical practice and have proved to be useful in predicting the genotype of the resulting pregnancy, although many problems remain to be resolved. We describe these methods based on our original experience of approximately 1000 PGD cycles, which currently exceeds the overall experience in the rest of the world. The major emphasis is on the illustrative material, which may provide a working manual for the establishment and performance of PGD in the framework of IVF and genetic services.

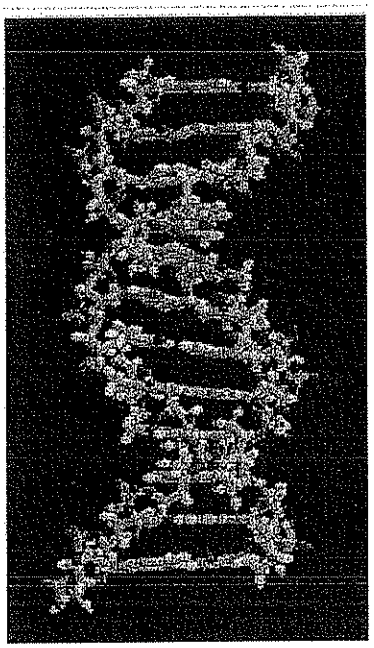
Although PGD was initially offered to couples at high risk for having children with single-gene disorders, more than half of the procedures have been performed for age-related aneuploidies. The list of conditions for which PGD was performed is being extended rapidly. Many centers at present concentrate on chromosomal aneuploidies and sex selection, using the fluorescence *in situ* hybridization (FISH) technique, while others also perform single-cell analysis by the polymerase chain reaction (PCR) for single-gene disorders. Additionally, some centers use PGD for focusing on a particular group of conditions, such as thalassemias in Cyprus.

As mentioned, the majority of PGD procedures have been performed for chromosomal disorders. Overall, more than 1000 clinical cycles have been performed, resulting in approximately 300 pregnancies, from which more than 200 healthy babies have already been born. Most of these cycles (approximately 800 IVF cycles) were performed in our center, using sampling of the first and second polar bodies (PB1 and PB2) for patients of advanced maternal age, demonstrating the clinical significance of the procedure for improving the chances of

EXHIBIT 2

Introduction to genetics

From Wikipedia, the free encyclopedia



A section of DNA; the sequence of the plate-like units (nucleotides) in the center carries information.

Genetics studies how living organisms inherit features from their ancestors – for example, children often look like their parents. Genetics seeks to identify which features are inherited, and explain how these features are passed from generation to generation.

In genetics, a feature of an organism is called a "trait". Some traits are features of an organism's physical appearance, for example, a person's eye-color, height or weight. There are many other trait types, and these range from aspects of behavior to resistance to disease. Traits are often inherited, for example tall and thin people tend to have tall and thin children. Other traits come from the interaction between inherited features and the environment. For example a child might inherit the tendency to be tall, but if there is very little food where they live and they are poorly nourished, they will still be short. The way genetics and environment interact to produce a trait can be complicated: for example, the chances of somebody dying of cancer or heart disease seem to depend on both their family history and their lifestyle.

Genetic information is carried by a long molecule called DNA which is copied and inherited across generations. Traits are carried in DNA as instructions for constructing and operating an organism. These instructions are contained in segments of DNA called genes. DNA is made of a sequence of simple units, with the order of these units spelling out instructions in the genetic code. This is similar to the order of letters spelling out words. The organism "reads" the sequence of these units and decodes the instruction.

Not all the genes for a particular instruction are exactly the same. Different forms of one type of gene are called different alleles of that gene. As an example, one allele of a gene for hair color could carry the instruction to produce a lot of the pigment in black hair, while a different allele could give a garbled version of this instruction, so that no pigment is produced and the hair is white. Mutations are random events that change the sequence of a gene and therefore create a new allele. Mutations can produce a new trait, such as turning an allele for black hair into an allele for white hair. The appearance of new traits is important in evolution.

Genetics glossary

DNA

A long molecule that looks like a twisted ladder. It is made of four types of simple units and the sequence of these units carries information, just as the sequence of letters carries information on a page.

Nucleotides

They form the rungs of the DNA ladder and are the repeating units in DNA. There are four types of nucleotides (A, T, G and C) and it is the sequence of these nucleotides that carries information.

Contents

Inheritance in biology

Genes and inheritance

Genes are inherited as units, with parents dividing out their genes to their offspring. You can think of this process like mixing two hands of cards, shuffling them, and then dealing them out again. Humans have two copies of each of their genes (*i.e,* two alleles) and when people reproduce they make copies of their genes in eggs or sperm, but only put in one copy of each type of

gene. An egg then joins with a sperm to give a child with a new set of genes. This child will have the same number of genes as its parents but for any gene one of their two copies will come from the father, and one from the mother.[1]

The effects of this mixing depends on the types (the alleles) of the gene you are interested in. If the father has two alleles specifying green eyes, and the mother has two alleles specifying brown eyes, all their children will get two alleles giving different instructions, one for green eyes and one for brown. The eye color of these children depends on how these alleles work together. If one allele overrides the instructions from another, it is called the *dominant* allele, and the allele that is overridden is called the *recessive* allele. In the case of a daughter with both green and brown alleles, brown is dominant and she ends up with brown eyes.[2]

Green eyes are a recessive trait.

However, the green eye color allele is still there in this brown-eyed girl, it just doesn't show. This is a difference between what you see on the surface (the set of observable traits of an organism, also called its phenotype) and which genes are in this organism (its genotype). In this example you can call the brown allele "B" and the green allele "g". (It is normal to write dominant alleles with capital letters and recessive ones with lower-case letters.) The brown-eyed daughter has the "brown eye phenotype" but her genotype is Bg, with one copy of the B allele, and one of the g allele.

Now imagine that this woman grows up and has children with a brown-eyed man who also has a Bg genotype. Her eggs will be a mixture of two types, one sort containing the B allele, and one sort the g allele. Similarly, her partner will produce a mix of two types of sperm containing one or the other of the two alleles. Now, when the alleles are mixed up in the offspring, these children have a chance of getting either brown or green eyes, since they could get a genotype of BB = brown eyes, Bg = brown eyes or gg = green eyes. In this generation, there is therefore a chance of the recessive allele showing itself in the phenotype of the children - some of them may have green eyes like their grandfather.[2]

Many traits are inherited in a more complicated way than the example above. This can happen when there are several genes involved, each contributing a small part to the end result. Tall people tend to have tall children because their children get a package of many alleles that each contribute a bit to how much they grow. However, there are not clear groups of "short people" and "tall people", like there are groups of people with brown or green eyes. This is because of the large number of genes involved; this makes the trait very variable and people are many different heights.[3] Inheritance can also be complicated when the trait depends on the interaction between genetics and the environment. This is quite common, for example, if a child does not eat enough nutritious food this will not change traits like eye color, but it could stunt their growth.[4]

Chromosome

A package for carrying DNA in the cells. They contain a single long piece of DNA that is wound up and bunched together into a compact structure. Different species of plants and animals have different numbers and sizes of chromosomes.

Gene

A segment of DNA. Genes are like sentences made of the "letters" of the nucleotide alphabet, between them genes direct the physical development and behavior of an organism. Genes are like a recipe or instruction book, providing information that an organism needs so it can build or do something - like making an eye or a leg, or repairing a wound.

Allele

The different forms of a given gene that an organism may possess. For example, in humans, one allele of the eye-color gene produces green eyes and another allele of the eye-color gene produces brown eyes.

Genome

The complete set of genes in a particular organism.

Genetic engineering

When people change an organism by adding new genes, or deleting genes from its genome.

Mutation

An event that changes the sequence of the DNA in a gene.

Inherited diseases

Some diseases are hereditary and run in families; others, such as infectious diseases, are caused by the environment. Other disorders are caused by a combination of hereditary and environmental factors.[5]

Diseases that are caused by a single allele of a gene and are inherited in families are called genetic disorders. These include diseases like Huntington's disease, Cystic fibrosis or Duchenne muscular dystrophy. Cystic fibrosis, for example, is caused by mutations in a single gene called *CFTR* and is inherited as a recessive trait.[6] Other diseases are influenced by genetics, but which alleles a person gets from their parents only changes their risk of getting a disease. Most of these diseases are inherited in a complex way, with either multiple genes involved, or both genes and the environment being important.

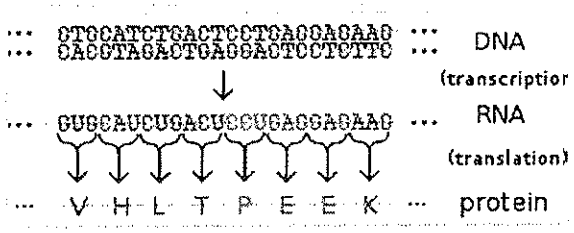
As an example, the risk of breast cancer is 50 times higher in the families most at risk, compared to the families least at risk. This variation is probably due to a large number of alleles. Each of them changes the risk a little bit.[7] Several of the genes involved have been identified, such as *BRCA1* and *BRCA2*, but not all of them. However, although some of the risk is genetic, being overweight, drinking a lot of alcohol, or not exercising, all increase the risk of this cancer.[8] A woman's risk of breast cancer is therefore the result of a large number of alleles and her environment, so it is very hard to predict.

How genes work

Genes make proteins

The function of genes is to provide the information needed to make molecules called proteins in cells.[1] Cells are the smallest independent parts of organisms: the human body contains about 100 trillion cells, while very small organisms like bacteria are just a single cell. A cell is like a miniature and very complex factory that can make all the parts needed to produce a copy of itself, which happens when cells divide. There is a simple division of labor in cells - genes give instructions and proteins carry out these instructions, tasks like building a new copy of a cell, or repairing damage.[9] Each type of protein is a specialist that only does one job, so if a cell needs to do something new, it must make a new protein to do this job. Similarly, if a cell needs to do something faster or slower than before, it makes more or less of the protein responsible. Genes tell cells what to do by telling them which proteins to make and in what amounts.

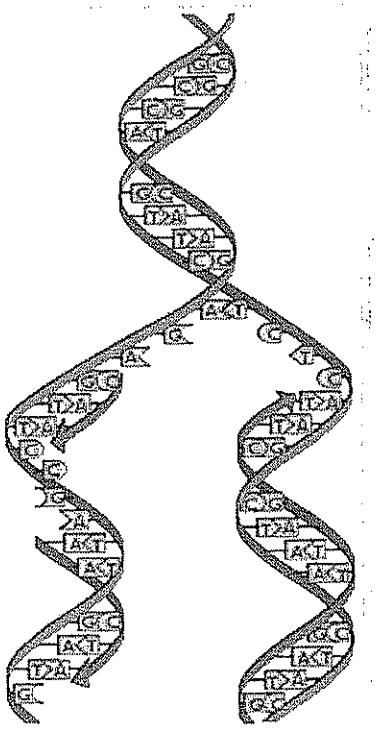
Proteins are made of a chain of 20 different types of amino acids. This chain folds up into a compact shape, rather like an untidy ball of rope. The shape of the protein is determined by the sequence of amino acids along its chain and it is this shape that, in turn, determines what the protein will do.[9] For example, some proteins have depressions in their surface that perfectly match another molecule, allowing the protein to bind to this molecule very tightly. Other proteins are enzymes, which are like tiny machines that can alter other molecules.[10]



Genes are expressed by being transcribed into RNA, and this RNA then translated into protein.

The information in DNA is held in the sequence of the repeating units along the DNA chain.[11] These units are four types of nucleotides (A,T,G and C) and the sequence of nucleotides stores information in an alphabet called the genetic code. When a gene is read by a cell the DNA sequence is copied into a very similar molecule called RNA (this process is called transcription). Transcription is controlled by other DNA sequences (such as promoters), which show a cell where genes are, and control how often they are copied. The RNA copy made from a gene is then fed through a structure called a ribosome, which translates the sequence of nucleotides in the RNA into the correct sequence of amino acids and joins these amino acids together to make a complete protein chain. The new protein then folds up into its active form. The process of moving information from the language of DNA into the language of amino acids is called translation.[12]

If the sequence of the nucleotides in a gene changes, the sequence of the amino acids in the protein it produces may also change - if part of a gene is deleted, the protein produced will be shorter and may not work any more.[9] This is the reason why different alleles of a gene can have different effects in an organism. As an example, hair color depends on how much of a dark substance called melanin is put into the hair as it grows. If a person has a normal set of the genes involved in making melanin, they make all the proteins needed and they grow dark hair. However, if the alleles for a particular protein have different sequences and produce proteins that do not do the job correctly, no melanin will be



DNA replication. DNA is unwound and nucleotides are matched to make two new strands.

produced and the hair will be white. This condition is called albinism and the person with this condition is called an albino.[13]

Genes are copied

Genes are copied each time a cell divides into two new cells. The process that copies DNA is called DNA replication.[11]. It is through a similar process that a child inherits genes from its parents, when a copy from the mother is mixed with a copy from the father.

DNA can be copied very easily and accurately because each piece of DNA can direct the creation of a new copy of its information. This is because DNA is made of two strands that pair together like the two sides of a zipper. The nucleotides are in the center, like the teeth in the zipper, and pair up to hold the two strands together. Importantly, the four different sorts of nucleotides are different shapes, so in order for the strands to close up properly, an **A** nucleotide must go opposite a **T** nucleotide, and a **G** opposite a **C**. This exact pairing is called base pairing.[11]

When DNA is copied, the two strands of the old DNA are pulled apart by enzymes which move along each of the two single strands pairing up new nucleotide units and then zipping the strands closed. This produces two new pieces of DNA, each containing one strand from the old DNA and one newly made strand. This process isn't perfect and sometimes the proteins will make mistakes and put the wrong nucleotide into the strand they are building. This causes a change in the sequence of that gene. These changes in DNA sequence are called mutations.[14] Mutations produce new alleles of genes. Sometimes these changes stop the gene from working properly, like the melanin genes discussed above. In other cases these mutations can change what the gene does or even let it do its job a little better than before. These mutations and their effects on the traits of organisms are one of the causes of evolution.[15]

EXHIBIT 3

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UNITED STATES DISTRICT COURT
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GENESIS GENETICS INSTITUTE, LLC, of the State of Michigan, MARK R. HUGHES, NEW YORK UNIVERSITY SCHOOL OF MEDICINE and NEW YORK UNIVERSITY HOSPITALS CENTER, both corporations in the State of NEW YORK, ABC CORPS. 1-10, JOHN DOES 1-10,	
Defendants.	

DECLARATION OF CHAYA GROSSBAUM

CHAYA GROSSBAUM, being of full age, hereby declares as follows, pursuant to 28 U.S.C. Sec. 1746:

1. I have been a New Jersey resident all of my life with the exception of three years after my marriage in August, 2002 when my husband and I temporarily lived in Brooklyn, New York prior to our childbearing years. It was always our intention to move back to New Jersey when we began a family.

2. I was born in Morristown, New Jersey and went to the Morristown schools, except for the last two years of high school, when I went away to school. I met my husband when I was in high school in Morristown, New Jersey, and he was a student at the Rabbinical College of America, also in Morristown, New Jersey.

3. My parents have been New Jersey residents since before I was born. My husband was born and raised in Minnesota.

4. Our family plan was that when I had our first baby we would return to New Jersey. In pursuit of that plan: (a) I came under the care of Marla Scott, CNM and Judy Caruso, CNM at Midwives of Denville, Boonton, NJ; (b) delivered the baby, Rosie, at Saint Clare's Hospital-Denville Campus, Denville, New Jersey; (c) prior to the baby's birth, arranged for a pediatrician, Dr. Richard Dicker, whose practice was located at 10 Broadway in Denville, New Jersey.

5. In addition, when I was informed, two weeks after Rosie was born, that she was a cystic fibrosis baby, I arranged for consultation with the Cystic

Fibrosis Center at Morristown Memorial Hospital, Morristown, New Jersey, where Rosie has been a patient since her birth approximately six years ago.

6. The only reason for having contact with NYU Medical Center and its Fertility Clinic was the IVF services offered by that institution allowed a rabbi to oversee the IVF processes to confirm compliance with Jewish law.

7. My decision to undergo IVF with preimplantation genetic diagnosis was influenced by the following:

a. My religious leader, Rabbi Markowitz, suggested that I consult with a rabbi of greater authority, Rabbi Tendler, and he brought my husband and me to see Rabbi Tendler. Rabbi Tendler confirmed that an abortion for parenting a child affected by cystic fibrosis was not an option within the parameters of Jewish law and suggested that I investigate PGD. Rabbi Markowitz's brother-in-law was Rabbi Jacobowitz of New York, who then introduced us to NYU for PGD purposes.

b. The decision of my husband and I to undergo in-vitro fertilization with PGD analysis was made strictly based on our recognition that it would eliminate the risk of having a child affected with cystic fibrosis since early termination of the pregnancy was not an option. We certainly would not have spent our last penny ($20,000 in the bank) for IVF since I did not have a fertility issue, only an issue of protecting against a genetically malformed child. I made this choice clear to all concerned with the IVF process at NYU including Dr. Licciardi and the nurses, as well as Dr. Hughes. Although I was aware of the language of the consent form, it appeared to be minimized by the doctors and personnel at NYU as well as Dr. Hughes. In addition, amniocentesis and CVS did not seem relevant as abortion was not available.

9. As stated above, the cost of the process of undergoing PGD as well as IVF exceeded approximately $20,000, which was not covered by our insurance. Also, the process itself was not totally without inconvenience and considerable effort. There were numerous injections for enhancing my hormone development as well as the need for my husband to be involved with sperm donation.

10. I am aware that there is a claim by the Defendants in this case that my baby, Rosie Grossbaum, was born as a result of our failure to comply with abstinence requirements imposed by the Fertility Clinic in connection with the PGD and IVF procedures. I state in the strongest terms that at no time did we depart from the instructions of NYU with respect to abstinence from sexual intercourse or the times that hormone injections were to take place to enhance my fertility with respect to the IVF process. I do not have a recollection of the specific dates with regard to the instructions regarding abstinence, nor do I have in my possession anything in writing relative to that subject. However I do have the patient instructions for egg retrieval and instructions for semen collection for in-vitro fertilization provided by NYU. In particular, the instructions for collection at home, which we undertook, contemplated the use of special condoms. Over-the-counter condoms were not acceptable. The semen speciman was to be collected in special sterile condoms during intercourse, which we used. See copy of Instructions for Semen Collection for In Vitro Fertilization that was given to us by NYU and that is attached to this Declaration. Our commitment to the process was too great for us to disrespect whatever the instructions were for undergoing IVF/PGD.

11. With respect to Dr. Hughes, I was told and directed to call Dr. Hughes on the telephone by the Fertility Clinic personnel at NYU. My conversation with Dr. Hughes did include a comment by him that

the process was not 100%, but he led us to believe that his skills and experience had virtually eliminated the risk of our having a cystic fibrosis baby. He was quite enthusiastic about his experience with regard to PGD and minimized the statistical risk of a misdiagnosis. He sounded so successful that we came away with the impression that it was a very safe undertaking.

12. Following the initial telephone conversation with Dr. Hughes in March 2004, at no time did we have any further conversation with Dr. Hughes or anyone connected with Genesis Genetics. I received no further telephone communication or written communication from Dr. Hughes or Genesis Genetics and was not instructed to call Genesis Genetics or Dr. Hughes for further information or advice.

13. When the IVF procedure was being concluded, that is the laboratory analysis had been done in Detroit and Dr. Licciardi at NYU was informed that their studies had been complete, and we were ready for implantation of the approved embryos, I was called to NYU to immediately come in for the procedure. When we arrived at NYU, we spoke to Dr. Licciardi who advised that there were two embryos that were deemed suitable for implantation and inquired as to whether I wanted to have one or two implanted with the hope of pregnancy following. I was also told that the two embryos that were going to be implanted were labeled "carrier at worst." My only understanding of the significance of that term was that my baby would be in the same position that I and my husband were in, in that we were carriers. There was no indication, or even mention, that the embryos that were created and cells sent to Genesis Genetics were in any way of limited suitability for the studies to be made at Genesis Genetics. I was not told or any way advised that the risk of having a cystic fibrosis baby was higher because of something called allele dropout that impacted the ability of the laboratory to be certain of the accuracy of their reporting and consequently, that there would be a

greater risk of misdiagnosis. Also, it was never mentioned that it may be advisable to redo the IVF process (not that we would afford it) and give the laboratory an opportunity for further study of our mutations with the embryos not sent or with blood from family members.

14. The only resource person that we had at NYU for genetic counseling was Dr. Licciardi.

Pursuant to 28 U.S.C. Sec. 1746, I declare under penalty of perjury that the foregoing is true and correct.

Dated: February 14, 2011

Chaya Grossbaum

New York University School of Medicine
Program *for* In Vitro Fertilization, Reproductive Surgery and Infertility

INSTRUCTIONS FOR SEMEN COLLECTION FOR IN VITRO FERTILIZATION

Please abstain from ejaculation between two (2) to five (5) days prior to the day of egg retrieval. This abstinence period has been shown to result in the production of an optimal semen sample. To calculate this properly, it is suggested that you ejaculate on the day that your partner is instructed to take her hCG injection.

Collection at NYU Program for IVF:
If the egg retrieval falls on a weekday, you should arrive at 9:30 am, or at a time indicated by the nurse calling with instructions. All IVF procedures are performed at **The NYU Program for IVF located at 660 First Avenue on the fifth floor.** On weekends, scheduling for semen collection is arranged on an individual basis and a nurse will tell you the appointment time. Go to the reception desk and state that you are here to produce a sample for IVF. The receptionist will contact the lab and an embryologist will escort you to the collection room.

You will be given a sterile container to collect your specimen. Please write your full name and your partner's full name on the label and place it on the side of the container. The specimen should be obtained by masturbation - ejaculate directly into the container. If you wish to use lubricant, we will provide you with sterile mineral oil. **Do not use saliva, soap or any other lubricants, i.e. baby oil, since these substances may be toxic to the eggs and embryos.**

After producing the specimen in our collection room, notify the laboratory by pressing the button labeled "IVF Lab." Open the steel connecting door and an embryologist will then collect the container and the completed semen collection record. **It is imperative that you contact us one hour after collection either in person or by phone at (212) 263-8990, to assure that a second sample is not required.**

Collection at home:
If you cannot collect your semen by masturbation, please request a special sterile condom and a sterile specimen container from a nurse **before** beginning your IVF cycle. Over the counter condoms are not acceptable. The semen specimen is collected in the *special sterile* condom during intercourse. Seal the condom with the provided tie and place it in the specimen container. The sample must be delivered to our facility within one hour after ejaculation. Care must be taken to keep the sample in an upright position and close to body temperature, especially in the winter months

If you have stored frozen semen, the frozen specimen will be only used as a back up in case the fresh specimen does not yield a sufficient number of motile sperm for insemination or in case there are problems with producing a fresh specimen on the day of retrieval. The frozen sample will not be used as a replacement for a fresh sample.

If you have any questions, please contact an IVF nurse or embryologist at (212) 263-8990.

03/03

EXHIBIT 4

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Review

Molecular Diagnostics in Preimplantation Genetic Diagnosis

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Preimplantation genetic diagnosis (PGD) is a procedure that allows embryos to be tested for genetic disorders before they enter the uterus and before pregnancy has begun. Embryos obtained by *in vitro* fertilization undergo a biopsy procedure in which one or two cells are removed and tested for a specific disorder. If the cell is unaffected, the embryo from which it was taken is judged to be free of the disorder. The embryo can then be transferred to the uterus to initiate pregnancy. Couples whose children are at increased risk for a specific genetic disorder can benefit from PGD. Some of these couples may have affected family members or family ancestry that puts them at high risk for transmitting a particular disorder to their offspring. PGD is an alternative to prenatal tests such as amniocentesis or chorionic villus sampling and since it is performed before a pregnancy has begun, it may be more acceptable to couples who have either had an affected child, previous termination of pregnancy, or who have objections to termination of pregnancy.

PGD tests have largely focused on two methodologies: fluorescent *in situ* hybridization (FISH) and polymerase chain reaction (PCR). This review will focus on the use of PCR-based methodologies to diagnose single gene disorders in single cells; specifically describing the characteristics and limitations of single cell PCR and mutation detection strategies which have been developed for use in clinical PGD.

The hundreds of cycles of preimplantation diagnosis performed to date have resulted in the birth of several hundred healthy children.[1] As shown in Table 1, the genetic conditions for which PGD has been applied are numerous and the various methods used for diagnosis reflect the heterogeneity of causative mutations.

The first clinical application of PGD used a generic PCR protocol for gender determination to avoid the transfer of male embryos which have a 50% probability of being affected by an X-linked recessive disorder. Gender was determined in a single blastomere by a single round of PCR using primers for Y-chromosome specific repetitive DNA sequences. The presence of Y-specific PCR amplification products was indicative of a male embryo and the absence of products was scored as female.[2] Although this approach had some success, a misdiagnosis, presumably due to amplification failure, did occur and emphasized the challenges inherent in single cell analysis and, more specifically, the danger in relying on the absence of amplification to diagnose genotype.[3] Subsequently, PCR protocols for preimplantation gender determination were refined to include primer sets which simultaneously amplify sequences common to both sex chromosomes (for example single copy genes such as *ZFX/ZFY*,[4] *AMELX/AMELY*,[5]) and repetitive sequences such as DXZ1 and DYZ1.[6,7] Sequences common to the sex chromosomes are identical at the site of primer annealing but differ internally in terms of size or include minor polymorphisms. Despite these technical improvements, the majority of embryo sexing is now accomplished using fluorescent *in situ* hybridization (FISH) which is less prone to contamination and can also provide the copy number for each chromosome tested thereby potentially avoiding the transfer of common chromosome abnormalities such as triploidy or X-monosomy.[8,9]

Although FISH has largely superseded PCR for sex determination, the specific diagnosis of single-gene defects remains dependent on DNA amplification with PCR. In the case of X-linked disorders, testing of the specific gene has the added advantage of ensuring that all embryos free of the mutant gene can be selected for transfer, irrespective of gender.[10–12] The list of disorders and the particular mutation detection strategies used for PCR-based clinical PGD application are given in Table 1.

Materials and Methods

Essentially there are two laboratory components involved in PGD. The first involves the collection of diagnostic material for testing. This is usually performed in a clinical

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Table 1. Strategies for PCR-Based Tests Used for Clinical Preimplantation Genetic Diagnosis

Method	Disorder to be diagnosed	Mutation type
Single PCR, agarose gel (+/− Y band)	X-linked disorders[2]	Various (gender determination to exclude hemizygotes)
Nested PCR, agarose gel (+/− X/Y)	X-linked disorders[6]	Various (gender determination to exclude hemizygotes)
Nested PCR, heteroduplexing	Cystic fibrosis[22,38,44,75]	3 bp deletion (ΔF508)
	Tay-Sachs disease[111]	4 bp insertion
Nested PCR, allele-specific amplification	RhD blood typing[3]	+/− RhD gene determines Rh status
	Myotonic dystrophy[127]	Expansion of (CTG)n trinucleotide repeat
Nested PCR, restriction enzyme	Cystic fibrosis,[23] Beta thalassemia,[83] Marfan syndrome,[107] Epidermolysis Bullosa,[100] Lesch-Nyhan syndrome,[101] Sickle cell anemia,[102] Fanconi's anemia,[103] Ornithine transcarbamylase deficiency,[104]	Various point mutations
	Spinal muscular atrophy[108–110]	Deletion. Distinguish between gene and pseudogene
Nested PCR, restriction enzyme (2 mutations in 1 fragment)	Skin fragility ectodermal dysplasia syndrome[66]	Allows detection of ADO
Whole genome amplification and comparative genome hybridization	Aneuploidy screening[96]	NA
Whole genome amplification (PEP)	Familial adenomatous polyposis coli[60]	Multiple analyses from each sample
Nested PCR, linked markers	Duchenne muscular dystrophy[10,125]	Exon deletions
	Ornithine transcarbamylase deficiency[104]	Point mutation (linked marker for ADO detection)
Nested PCR, SSCP	Familial Adenomatous Polyposis Coli[60]	Point mutation
Nested PCR, direct cycle sequencing	Skin fragility ectodermal dysplasia syndrome[66]	Point mutations (cycle sequencing to confirm restriction digest)
Nested PCR, DGGE	Beta thalassemia[115]	Point mutations
Heminested PCR, site specific mutagenesis	Retinitis pigmentosa[99]	Point mutation
	Ornithine transcarbamylase deficiency[104]	Point mutation
Heminested PCR, allele dependent length polymorphism	Retinitis pigmentosa[99]	Point mutation
Nested multiplex PCR (including linked markers)	Marfan syndrome[119]	Unknown mutation
	Epidermolysis Bullosa[100]	Monitor allele dropout
	Beta thalassemia[83]	
Nested multiplex PCR (including linked and non-linked markers)	Sickle cell anemia,[41] hemophilia B,[41] cystic fibrosis,[41] Gaucher disease,[61] Long chain 3-hydroxyacyl-CoA dehydrogenase deficiency[61]	Monitor allele dropout and contamination
Fluorescent PCR, allele size (fragment analysis)	Huntington disease[32]	Expansion of (CAG)n trinucleotide repeat
	Cystic fibrosis[106]	3 bp deletion
	Myotonic dystrophy[55]	Expansion of (CTG)n trinucleotide repeat
	Fragile X syndrome[128]	Expansion of (CGG)n trinucleotide repeat
Fluorescent PCR, SSCP	Medium chain acyl CoA dehydrogenase deficiency[76]	Point mutation
Fluorescent PCR, ARMS	Spinal muscular atrophy[77]	Exon deletion in gene but not pseudogene
Fluorescent PCR, restriction analysis	Congenital adrenal hyperplasia,[78] osteogenesis imperfecta,[105] medium chain acyl CoA dehydrogenase deficiency,[33] Sickle cell anemia[34]	Point mutations
Fluorescent PCR, restriction analysis (2 mutations in 1 fragment)	Beta thalassemia[34]	Point mutations, small deletion
Multiplex Fluorescent PCR	Beta thalassemia[34]	Point mutations, small deletion
Multiplex Fluorescent PCR (including unlinked marker)	Myotonic dystrophy[130]	Expansion of (CTG)n trinucleotide repeat/ contamination control
Multiplex Fluorescent PCR (including linked marker)	Medium chain acyl CoA dehydrogenase deficiency[76]	Maternal mutation unknown
Fluorescent PCR, linked markers only	Fragile X syndrome[120]	Expanded (CGG)n repeat (refractory to PCR)
	Marfan syndrome[121]	Unknown mutation
	Charcot Marie Tooth disease[45]	Gene duplication
	Cystic fibrosis[123]	Heterogeneous mutations

in vitro fertilization (IVF) laboratory under sterile conditions. A set of micromanipulators linked to an inverted microscope with contrast optics and facilities for extended embryo culture are the minimum essential requirements to carry out diagnostic biopsy procedures. The second step involves the diagnostic test itself, which can be performed in a region of the IVF laboratory, an adjacent laboratory equipped to perform molecular analyses or in a completely separate dedicated molecular genetics laboratory equipped to process single cell sam-

Table 2. Strategic Considerations for PCR Analysis of Diagnostic Material Biopsied at Different Developmental Stages for Preimplantation Genetic Diagnosis

Stage	Advantages	Disadvantages
Oocyte (1st polar body)	Removal has no effect on embryo development Increased time to perform PCR analysis prior to transfer	Only 1 cell available for analysis Increased risk of diagnostic error Maternally inherited disease only Fewer embryos for transfer (recombination)
Zygote (1st and 2nd polar body)	2 cells for analysis (greater accuracy/reliability) Removal has no effect on embryo development Increased time to perform PCR analysis prior to transfer	Maternally inherited disease only Narrow time window to complete biopsy
Cleavage stage (blastomeres)	Diagnosis of maternally/paternally inherited disorders Large body of clinical data available 2 cells available for analysis (greater accuracy/reliability)	Chromosomal mosaicism present Selection of nucleated blastomere is critical
Blastocyst (trophectoderm)	Sample multiple cells (eliminate PCR failure/ADO) Trophectoderm sampled rather than inner cell mass Embryo quality preselected Higher implantation rate/lower multiple gestation rate	Time for PCR analysis may be limited Cells may not be representative of embryo Fewer embryos for analysis Limited clinical data available

ples. Minimum requirements include a PCR preparation area (usually a small, dedicated flow hood), dedicated PGD reagent storage facilities, thermal cycler, and access to the necessary post-PCR mutation detection apparatus. The critical component of the diagnostic step is to minimize the level of contamination and a number of possible laboratory designs and procedures may fulfill this requirement.

Theoretically, diagnostic material can be collected at any developmental stage between the mature oocyte and blastocyst. To date, four distinct stages have been targeted; metaphase II oocyte, zygote, cleavage stage embryo, and blastocyst. The four stages dictate different diagnostic strategies, each with its own limitations. The different technical approaches required to obtain the material and the material itself can affect the success rate of the procedure. The strengths and limitations of each approach are summarized in Table 2.

Each of the biopsy methods involves at least two steps; the first step being to breach the zona pellucida while the second involves the removal of cellular material (be that polar body, blastomere, or trophectodermal cells). Zona breaching can be achieved mechanically (by means of a sharp microneedle), chemically (using acidified Tyrodes solution, pH 2.2), or by thermal ablation (using a non-contact laser). Removal of cellular material is generally carried out using a glass micropipette attached to a pneumatic or hydraulic based suction system.[13]

At present, polar body biopsy in combination with PCR based assays is performed almost exclusively by one group[14,15] while the majority of PGD centers[16] obtain genetic material for PGD by cleavage stage biopsy on the third day following insemination when the embryo has between 6 and 10 cells. At this stage, blastomeres are believed to be totipotent and embryo survival and metabolism appears to be unaffected by biopsy.[17] While blastocyst biopsy appears to be a promising approach[18–20] its clinical utility for PGD has yet to be demonstrated in clinical practice.

Diagnostic Methods

The success of PCR in amplifying small quantities of DNA to a level at which they can be visualized and subjected to further genetic analysis has made the technique one of the most important diagnostic techniques in the modern molecular laboratory. Application of PCR protocols to single cell analyses has proved to be challenging but ultimately highly successful, and remains the only means of detecting specific mutant alleles in human preimplantation embryos. The limited amount of template DNA (approximately 7 pg) available in a single diploid cell leads to a number of problems which are rarely, if ever, observed in routine diagnostic PCR (in which a starting amount of DNA template of at least 10 ng is usually available). Problems frequently encountered include an increased incidence of detectable contamination, amplification failure, and extreme preferential amplification of one allele or complete absence of one allele (allele drop-out) in heterozygous samples.

Characteristics of Single Cell PCR (SCPCR)

Amplification Efficiency

Amplification efficiencies at the single cell level are generally lower than those encountered during the routine PCR of DNA samples in which the amount of starting template may be larger by several orders of magnitude. Reduced amplification efficiency can be the result of many problems encountered between sample collection and the PCR procedure itself. Operator problems such as cell loss during the delicate process of cell transfer to the tube or spontaneous cell lysis before the cell entering the tube contribute to amplification failure or reduced amplification efficiency. Intrinsic factors such as anucleate or degenerating cells with concomitant absence or degradation of DNA respectively are more difficult to control. Indeed, blastomeres from poor quality embryos yield lower amplification efficiencies than their high quality counterparts[21,22] underlining the importance of blas-

tomere selection during embryo biopsy. Following successful transfer of a high quality nucleated cell, the cell lysis protocol used also influences amplification success. Consecutive rounds of freezing and thawing in distilled water or boiling do achieve cell lysis[23] but the use of either alkaline or proteinase based lysis buffers has proved more effective.[24–29] Nevertheless, there is no consensus as to which lysis buffer is the most effective.[16]

Contamination

With only one or two DNA molecules present per haploid (second polar body, oocyte, or sperm) or diploid (blastomere or first polar body) cell respectively, extraneous DNA can easily lead to a misdiagnosis in clinical PGD. Contamination is an omnipresent threat in any molecular diagnostics laboratory but the large number of PCR cycles required for detectable amplification in combination with a single genome starting template exacerbates this threat. A series of stringent experimental practices can be implemented to counter contamination but there is no guaranteed method of eliminating sporadic contamination. Sources of contamination are numerous since DNA (particularly in the form of previously amplified PCR products[30]) can exist in aerosol form and, as such, is likely to be present on all exposed laboratory surfaces. Such "carry-over" contamination caused by the inadvertent amplification of PCR products generated in previous experiments is a cumulative problem and probably the most significant contamination threat in single cell PCR. To address this problem single cell reactions should be set up in a room designated for this purpose (pre-PCR area) and physically separated from the area in which PCR product analysis occurs (post-PCR area). Pre-PCR areas (including the cell preparation area, the reagent preparation area, and the PCR set-up area) kept under constant positive pressure can prevent the entry of contaminants but much of the cellular and PCR product contamination is introduced by human traffic. For this reason, dedicated gowns, gloves, overshoes, caps, and masks should be worn and remain in this room, together with dedicated equipment such as tubes, racks, and pipettes. Ideally, a unidirectional work flow prevents the re-introduction of items from a post-PCR area into a pre-PCR area. Filtration and autoclaving of reagents, incubation of component reagents of the reaction mix with restriction enzymes to destroy any PCR product (for example exonuclease III, *Alu* I, *Hae* III, and *Hinf* I)[31–34] or the use of a mineral oil overlay to provide a physical barrier against contamination may be of some value but the introduction of additional components into any clean system may be counterproductive and could potentially reintroduce contaminants. Routine decontamination of work surfaces and equipment using 10% bleach[35] or exposure to ultraviolet light to destroy DNA is also recommended. Unfortunately, no single strategy can be considered to be 100% effective or render the continuous monitoring of contamination levels obsolete. For this reason all PCR reagents, cell washing, and lysis solutions should be rigorously tested for contamination before any clinical diagnostic application.

Another measure for contamination control which has been used extensively in infectious disease screening by sensitive PCR but not yet in PGD is post-PCR sterilization. One method uses uracil DNA glycosylase (UDG) to cleave uracil bases from PCR products in which dUTP is substituted for dTTP in the PCR mix. In this way the action of DNA polymerase is blocked exclusively with carry-over contamination products but not native DNA.[36] A different technique uses isopsoralen which binds to PCR products such that photoactivation following amplification damages the DNA strand preventing it from functioning as a template in subsequent PCRs.[37]

Cellular DNA from sperm or maternal cumulus cells (both of which may be present on the zona pellucida of the human embryo) is another potential source of contamination but can be largely eliminated by means of precautions in the IVF laboratory. All cumulus cells must be carefully removed before biopsy and the embryo checked under an inverted microscope. Moreover, the use of intracytoplasmic sperm injection (ICSI) a technique used to introduce a single sperm into the cytoplasm of the oocyte has circumvented the problems caused by supernumerary sperm which frequently bind to the zona pellucida in large numbers following standard insemination techniques.[38] The biopsied blastomeres themselves should be washed through a series of fresh drops of holding medium known to be contamination-free before transfer to the PCR tube. The commonly used precautions against contamination are listed in Table 3.

Allele Dropout

Another problem unique to single cell PCR is that of allele dropout (ADO), a phenomenon whereby only one of the two alleles present is successfully amplified.[39–41] ADO is only detectable when heterozygous alleles are present but appears to be indiscriminate, in that the allele successfully amplified is random (even when only differing by a single nucleotide).[42] ADO remains the biggest obstacle to accurate and efficient PGD for single gene disorders and the severity of its consequences is closely linked to the mode of inheritance of the disorder under test. For autosomal recessive conditions when both partners are carrying the same mutation, ADO should not, in the absence of contamination, result in the transfer of an affected embryo. However, the number of embryos available for transfer will decrease as the ADO rate increases, potentially reducing the likelihood of pregnancy. In such cases, there is some reassurance in the calculation that a 10% allele dropout rate would only result in the exclusion of, on average, 2.5% of embryos for which a diagnosis was successfully made (based on a 90% amplification rate). In contrast, for compound heterozygous or autosomal dominant conditions, the consequences of ADO can be catastrophic, as misdiagnosis and subsequent transfer of affected embryos can occur.[43] Indeed ADO is the most likely cause of reported errors in PGD of cystic fibrosis in which affected compound heterozygote embryos were misdiagnosed as carrier embryos because the analysis used could only detect one of the inherited mutations.[9,44]

Table 3. Precautions against Contamination in Single Cell PCR

Type of contamination	Precautions
Operator contamination (can be cellular or product contamination; see below)	Elimination or reduction Protective clothing: gloves (close fitting), cap, overshoes, gown Frequent changes of gloves Operator technique Detection DNA Fingerprinting (incorporate informative polymorphic markers)
Product contamination (PCR products from previous reactions also known as "carry-over" contamination)	Elimination or reduction Dedicated equipment (PCR machine, pipettes, tubes) Dedicated reagents (all solutions) Filtration of reagents Filtered pipette tips Positive displacement pipettes Aliquot all reagents One-time use of tips and reagent aliquots UV irradiation of preparation area/equipment/reagents Autoclaving equipment and reagents Restriction enzyme digestion of PCR master mix (component reagents) Switch from nested PCR to FPCR Geographical separation of pre-PCR/PCR and post-PCR activities Preparation of PCR reagents in laminar flow Decontamination of surfaces/equipment with 10% bleach Post-PCR sterilization (dUTP and UDG/isopsoralen) Purchase reagents as ready-made 'molecular biology grade' solutions Reduce number of tube-opening events Mineral oil overlay Detection Switch from nested PCR to FPCR Use of multiple negative controls (cell wash blanks and reagent blanks) Test all component reagents before clinical case
Genomic DNA (gDNA) contamination (eg, DNA used for positive controls/assay development)	Elimination or reduction Isolate procedures involving gDNA (eg, no gDNA in reagent prep room)
Cellular contamination (eg, Maternal cells (cumulus), paternal cells (sperm), embryonic material (from different embryos) or operator cells (epithelial))	Elimination or reduction Rinse embryo thoroughly (to remove cumulus cells) Exclusive use of Intracytoplasmic sperm injection for fertilization (to prevent supernumerary sperm exposure) Wash blastomere thoroughly Change micropipettes if a cell lyses during biopsy or dish-to-tube transfer Detection DNA "fingerprinting" (incorporate informative polymorphic markers) Wash blank controls

The frequency of allele dropout reported in the literature varies widely and has been reported to be as high as 25% in a clinical PGD case.[45] This figure could be considered unacceptably high, but the concept of an acceptable ADO rate is meaningful only when parameters relevant to the PGD case have been assessed. For example, a more accurate ADO rate can be established as more cells are analyzed and a higher ADO rate tolerated with contamination rates close to zero in combination with diagnosis of a homozygous recessive mutation.

Reports suggest that blastomeres generally exhibit a greater ADO rate than polar bodies, lymphocytes, or fibroblasts[41,46] although such differences have not been unanimously reported.[47] Observations that amplification rates are generally lower for blastomeres than other cell types even when a nucleus is present[48,49] and the detection of haploidy in an estimated 7 to 15% of blastomeres provide further evidence for a cell-specific effect on the observed frequency of ADO.[50,51]

The origins of ADO remain elusive but experimental data supports the causative factors being suboptimal PCR conditions and/or incomplete cell lysis. Adequate denaturation is essential for amplification of both alleles as demonstrated by a reduction in ADO when the denaturation temperature is increased in the first cycles of PCR.[27]

ADO could also arise from DNA deterioration or damage such as strand breaks caused by endogenous nucleases. As with reduced amplification efficiencies, increased ADO is noted in degenerating cells presumably the result of strand-specific DNA degradation.[52] Additionally, access to the target genomic sequence by the primers and *Taq* polymerase may be restricted by, for example, adjacent G/C rich regions reducing denaturation efficiency or differing degrees of folding perhaps related to the stage of the cell cycle.[27] Whatever the exact cause, ADO likely arises in the initial cycles of the primary PCR before the number of target molecules is

increased by the process of amplification. Evidence for this suggestion comes from experiments in which different proportions of two separate populations of single cells (each homozygous for a different sized triplet repeat sequence) are mixed, demonstrating that the minority allele is undetectable when the starting template ratio is less than one in four cells.[53]

Allele dropout observed during conventional nested PCR with ethidium bromide detection comprises both extreme preferential amplification, in which the PCR product from one allele is present but at extremely low levels, and true allele dropout (in which one allele is either absent or has totally failed to amplify). Enhanced detection methods such as the use of fluorescent primers[40] and SYBR green I staining[54] have shown that a proportion of observed ADO is due to extreme preferential amplification. A fourfold reduction in ADO for both lymphoblasts and blastomeres has been reported after switching from an ethidium based protocol to one using fluorescence primers.[55] However a significant proportion of true ADO exists even using fluorescent PCR.[33,42] Such observations from single cell fluorescent PCR reinforce the need for cut-off values to distinguish background noise, contamination, extremely low amplification, preferential amplification, and allele dropout. Examples of preferential amplification and allele dropout from samples of single heterozygous cells are shown in Figure 1.

The use of alkaline lysis buffer or lysis buffer containing proteinase and detergent also seems to be beneficial in reducing ADO[24–29] although there is no consensus as to which lysis buffer to use.[16] Indeed, two reports offered dramatically different conclusions with one favoring proteinase K[28] and the other favoring alkaline lysis buffer.[29]

Protocols that rely on reverse transcription of abundant mRNA molecules followed by PCR (RT-PCR) and subsequent mutation analysis have been proposed as a means of reducing amplification failures and ADO since multiple targets should not be subject to allele specific amplification failure. Single cell expression assays have been developed for the diagnosis of Marfan[56] and Lesch-Nyhan[57] syndromes. Such assays could prove valuable for genes that are expressed at the cleavage stage, provided that they are not subject to genomic imprinting and that residual maternally-derived transcripts from the oocyte or alternatively spliced products[57] do not confuse the diagnosis.

Several other methods of decreasing ADO include the use of restriction enzymes before PCR to shorten genomic template strands (presumably making them more accessible to the polymerase enzyme during the first few cycles of PCR)[58] and the use of *Taq*/*Pwo* polymerase mixture (perhaps because of the proof-reading ability of *Pwo* polymerase).[59]

In addition to reducing ADO, strategies have been proposed to increase the detection of ADO. One such strategy is the use of linked markers[46,60,61] which simultaneously controls for contamination.[62] Use of one or two linked markers reduces undetected ADO by approximately 50% and 75% respectively and with three linked markers ADO is virtually always detected.[46] The use of linked markers carries considerable advantages not only

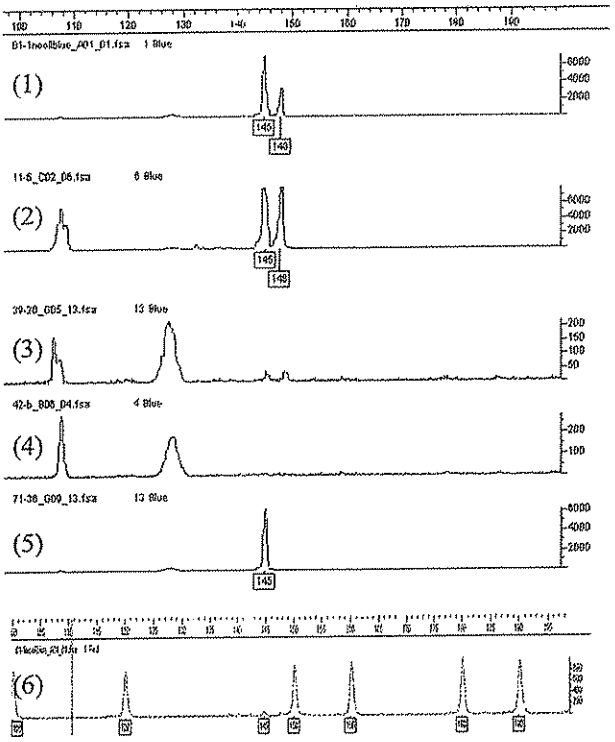

Figure 1. Genotyping of single heterozygous cells after fluorescent PCR. **Lanes 1, 2, 3, 5:** Single lymphoblast cells heterozygous for deltaF508 mutation (3-bp deletion) in cystic fibrosis. **Lane 1** demonstrates preferential amplification of the deleted allele (145 bp). **Lane 2** shows equivalent amplification from both alleles. **Lane 3:** Scored as amplification failure. Note the extremely low peaks in **lane 3** (corresponding to peaks at 145 and 148 bp) considered technical artifacts in view of the extremely low signal amplitude and proximity to a strong positive lane. **Lane 4:** Negative control (wash drop blanks). No amplification observed. **Lane 5:** Allele dropout in which the wild-type allele (148 bp) has failed to amplify to detectable levels. **Lane 6:** ROX-labeled size standard with peaks at 100, 120, 150,160, 180, and 190 bp. This size standard is labeled with a red fluorescent dye and is added to all samples to allow accurate sizing in each lane. **Lanes 1–5** are shown with the size standard trace removed for clarity. The *y* axis for each trace represents units of fluorescence and the *x* axis represents sizing (in bp) according to the internal size standard. All PCR products were generated using FAM-labeled primer and identified using an ABI3100 DNA analyzer with Genescan software.

from the point of view of reducing the possibility of misdiagnosis, but also by potentially increasing the number of embryos available for transfer.[63] For example, in a homozygous recessive condition, carrier embryos could still be transferred even when the normal allele appears to be absent due to ADO, but a linked marker (or markers) is present. However, the identification and work-up of reliable informative linked markers can be labor intensive and may not always be cost effective for all diseases, particularly when the patient population is very small.

Another strategy used to increase ADO detection is special design of the PCR assay itself. For disorders in which a triplet repeat expansion (which is refractory to PCR) is the disease causing DNA sequence change, an assay based on detection of two normal sized triplet repeat alleles will prevent transfer of affected embryos when the parental alleles are informative. In addition, assays in which a single amplified fragment encompasses both mutations of a compound heterozygous condition should always allow the detection of allele

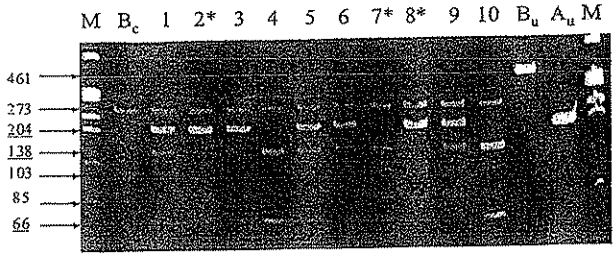

Figure 2. Use of internal restriction digestion control to avoid misdiagnosis. The PCR reaction generates a 204-bp product from the *LAMA3* gene and a 461-bp product from the *LAMB3* gene (which is used as an internal control). Digestion with *Dde* I cleaves the *LAMB3* product into fragment sizes of 273, 103, and 85 bp (B_c) and cleaves only the *LAMA3* allele that contains the R650X mutation (into fragment sizes 138 bp and 66 bp). M, Marker 1-kb DNA ladder (ϕX174 DNA/*Hae* III marker, Promega Corporation). **Lanes 1–10:** Single lymphocytes heterozygous for R650X mutation in *LAMA3* gene. A_u and B_u, uncut *LAMA3* and *LAMB3* PCR products, respectively. Allele dropout (*) is apparent in **lanes 2, 7 and 8.** The internal digestion control prevents an affected cell being misdiagnosed as unaffected (as a consequence of failed *Dde* I digestion) since the *LAMB3* product remains undigested (B_u) at 461 bp.

dropout in an affected blastomere[24,64–66] (Figure 2). In such cases, ADO of the wildtype allele in carrier embryos will result in a restriction pattern suggesting homozygosity for one particular mutation. This result is not possible when the parents carry different mutations. Such a pattern in a clinical diagnosis would result in rejection of that particular embryo for transfer since it would be impossible to distinguish between an unaffected carrier and an affected compound heterozygous embryo.

The existence of ADO and contamination (which may have been responsible for a serious misdiagnosis of myotonic dystrophy) prompted the directive to test two cells from each embryo regardless of the mode of inheritance.[67] Certainly, the probability of ADO affecting the same allele in both cells in independent reactions is low.[63] In a mouse embryo model, dual blastomere biopsy combined with independent blastomere analysis improved preimplantation diagnostic reliability dramatically for a dominant[68] condition but only slightly when the inheritance was recessive.[69] Whereas increasing the DNA template threefold is not effective in reducing ADO,[42] the risk of error due to ADO can be virtually eliminated if more than four cells are taken and independently analyzed.[70] Although blastocyst biopsy could make this approach feasible, the routine removal of four cells from cleavage stage embryos would be unacceptable in terms of the negative impact on subsequent embryo development. If stringently applied, even a two-cell policy would be dramatically affected by suboptimal embryo development on day 3, instantly reducing the cohort for biopsy and ultimately the number of potentially unaffected embryos available for transfer. A recent retrospective analysis showed that implantation rates of biopsied embryos were equivalent regardless of whether one or two cells had been removed[67] but so far no prospective randomized studies have been performed to test this hypothesis. A summary of the methods used for the reduction and detection of ADO is shown in Table 4.

PCR Strategies

Nested PCR

Using a conventional ethidium bromide-based detection system, around 50 to 60 cycles of PCR are required to obtain detectable products from unique sequences. Since the enzyme *Taq* polymerase incorporates mistakes after approximately 40 cycles, aspecific products appear with such large numbers of cycles. This problem led to the development of nested PCR in which two sequential rounds of PCR are used to improve sensitivity and specificity when amplifying unique sequences from single cells.[71,72] The primary PCR generates DNA fragments encompassing the mutation site but which are insufficient in number to be visualized. PCR products from the first reaction are transferred to a new PCR tube and are amplified to detectable levels using a different set of primers situated internally to the first. This strategy enhances the specificity of PCR, as well as reducing the risk of carry-over contamination for subsequent primary amplifications, as the secondary product cannot be amplified by the outer set of primers. However, the threat of contamination from primary PCR products in the first round PCR and secondary products in the second round PCR remains.

Fluorescent PCR

Fluorescent PCR (FPCR) is fast becoming the method of choice for laboratories performing single cell PCR for a number of reasons. Compared with nested PCR, FPCR combines increased sensitivity and throughput, shorter turnaround time,[73] and superior precision in fragment sizing.[62] The use of a laser system to perform automated fragment analysis with various fluorescent molecules, each with their own unique wavelength of emitted light, allows simultaneous discrimination of unrelated, similarly sized products. Furthermore, the thousandfold increase in sensitivity[74] compared with ethidium bromide allows a single round of PCR, potentially avoiding the contamination which can result from multiple tube openings.

The accuracy of automated fragment analysis enables, for example, a deletion of 3 bp in the ΔF508 mutation causing cystic fibrosis, to be clearly differentiated from the normal allele after fluorescent PCR (Figure 1) removing the need for either heteroduplex analysis[75] or lengthy conventional electrophoresis using a high resolution gel. Several different instruments are available for such analyses and the technique has been successfully applied to PGD development and clinical cases in many laboratories.[55,62,73] Fluorescent PCR is also compatible with many established forms of mutation analysis such as SSCP,[76] ARMS,[77] and restriction enzyme digestion.[78]

Multiplex PCR

By using combinations of unrelated primer sets in one PCR assay (multiplex PCR) it is possible to amplify multiple loci simultaneously and attempt to overcome the limitations of the single cell.[62,79–81] Providing there is no

Table 4. Strategies for the Reduction and Detection of Allele Dropout (ADO)

Action/measure	Reduce/detect ADO	Mechanism	Potential problems/disadvantages
Use of any lysis buffer	Reduce	Protein removal/DNA accessibility/destroys endogenous nucleases (fewer DNA strand breaks)	Quality control of additional reagents
Choice of lysis buffer	Reduce	As above	Quality control of additional reagents
Increase denaturation temperature in first ten cycles of PCR	Reduce	Accessibility of DNA, complete denaturation of DNA strands	Taq polymerase failure due to prolonged exposure to high temperature
≥2 cells taken from cleavage stage embryo (analyzed together)	Reduce	Increase starting template reduces probability of ADO	Possible detrimental effects of 2 cell biopsy
Reverse-transcriptase PCR	Reduce	Increased starting template	Prone to contamination/ presence of maternal transcripts/imprinted genes will exhibit ADO
Restriction enzyme digestion prior to PCR	Reduce	Shortens genomic DNA template strands—facilitating primer-template annealing	Limited data available
Use of Taq/Pwo-polymerase mixture	Reduce	Proof-reading activity?	No data available for single cells
Blastocyst biopsy (>2 cells)	Reduce/detect	Increase starting template reduces probability of ADO	Reduced embryo cohort at blastocyst stage
≥2 cells taken from cleavage stage embryo (analyzed independently)	Detect	Low probability of two independent analyses both exhibiting ADO	Possible detrimental effects of 2 cell biopsy
Fluorescent PCR	Detect	~1000 times more sensitive than ethidium. High sensitivity can identify preferential amplification	Equipment and reagent cost
SYBR green I fluorescent stain	Detect	~25 times more sensitive than ethidium bromide. High sensitivity can identify preferential amplification	Reagent cost
Same fragment PCR	Detect	Impossible to have ADO if fragment contains both mutations of interest. Either both alleles are detected or amplification failure is observed	Only a small proportion of compound heterozygous conditions have mutations within several hundred base pairs of each other
Diagnosis of two normal alleles	Detect	In the absence of contamination, the presence of two normal alleles indicates that a mutant (expanded) allele cannot be present	Maternal and paternal alleles must be informative
Use of linked markers	Detect	Low probability of ADO occurring at a series of different adjacent loci	Requires design of single cell duplex/multiplex PCR

interaction between unrelated primers or PCR products, the various loci should be amplified simultaneously within a single reaction. Each multiplex PCR need only be optimized for the combination of primers involved. Successful multiplex reactions enable the simultaneous assessment of numerous loci, with as many as 15 analyzed from larger DNA samples.[82] It may also be possible to assess similar numbers of loci in single cells but to date the maximum number of sequences amplified simultaneously from a single cell is seven using either conventional ethidium detection[12] or fluorescent PCR.[62] Unfortunately the problems of allele dropout and preferential amplification persist even with the FPCR approach.[40,80]

Multiplex PCR can also be used to detect ADO by simultaneous amplification of a disease causing mutation and an informative "linked" polymorphism. This is a particularly useful strategy when diagnosing dominant disorders, but has also been reported for a number of recessive disorders including cystic fibrosis,[41] β-thalassemia,[83] and medium chain acyl CoA dehydrogenase deficiency.[76] The probability of ADO affecting both mutation site and linked polymorphism is very low and consequently the mutant allele can almost always be detected.

Whole Genome Amplification

One of the most exciting developments in single cell analysis has been the evolution of protocols designed to amplify the entire genome from a single cell. Depending on the particular whole genome amplification (WGA) method used, a starting template of approximately 7 pg of DNA can be amplified up to 1000 times apparently overcoming the limitation of a single cell.[84] The technical difficulties sometimes associated with multiplex PCR, such as incompatibility of primer sets and problems distinguishing the various amplified products, are not encountered using WGA. Moreover WGA provides a supply of sample DNA that can be reassessed, allowing confirmation of diagnosis using the same or different methods or the analysis of other genes. The most commonly used method has been primer extension preamplification (PEP) which utilizes 15 base oligonucleotide primers of random sequence to initiate DNA synthesis throughout

the genome.[85] Reports estimate that between 70% and 96% of the genome is amplified between 30 and 1000 times.[84–86]

PEP has been used to develop PGD protocols for single cell analysis of Tay-Sachs disease,[87] cystic fibrosis,[88,89] hemophilia A,[90] and Duchenne muscular dystrophy,[91] but its clinical application has been limited. One problem is the length of time necessary, since PEP mandates an embryo transfer on day 4 post-fertilization at the earliest; however, a modified protocol has been reported that reduces the time required from >14 hours to 5 hours 30 minutes.[92] Nevertheless, PEP was successfully used for PGD in the dominant cancer syndrome familial adenomatous polyposis coli (FAP) allowing the subsequent amplification of two different fragments, one containing a mutation and the other an informative polymorphism.[60]

Another form of WGA is degenerate oligonucleotide primed PCR (DOP-PCR) which was designed to give general amplification of target DNAs at frequently occurring priming sites, without restrictions due to the complexity of the DNA or the species from which it was derived. It rests on the principle of priming from short sequences specified by the 3′ end of the oligonucleotides used, during the initial low temperature cycles of the PCR protocol. Since these short sequences occur frequently and are evenly distributed throughout the genome, amplification of target DNA proceeds at multiple loci simultaneously. Annealing of the specified 3′-most primer sequence is stabilized by the adjacent six bases of degenerate sequence which create a pool of 4096 primers of different sequence, as opposed to the single sequence of a nondegenerate primer. At the 5′ end of the primer is a further specified sequence which allows efficient annealing of primers to previously amplified DNA, enabling a higher annealing temperature to be used in later PCR cycles.[93] DOP-PCR amplifies a similar proportion of the genome to PEP, but to a much more significant level. A single cell subjected to DOP-PCR can provide enough DNA for over 100 subsequent PCR amplifications.[86] Furthermore sufficient DNA is produced to allow additional experimental procedures such as comparative genomic hybridization (CGH) for the detection of chromosome copy number in embryos[94,95]—an approach recently applied in clinical PGD.[96]

ADO rates after PEP and DOP-PCR are comparable to those obtained by direct amplification of single cell loci.[86] A significant drawback of WGA techniques is that amplification of repetitive DNA sequences, such as short tandem repeats, is error prone if performed on WGA products.[86,97] In some studies over 50% of fragments amplified differed from their expected size presumably due to the uniformly low temperatures needed for WGA which could allow slippage of the DNA chain during product generation.[86] Such errors would currently rule out the use of WGA for the clinical diagnosis of triplet repeat expansion diseases or diagnoses based on linkage analysis with STRs. The current difficulties associated with the WGA approach will no doubt be overcome because of the enormous potential of the technique to be combined with repeated simplex and multiplex PCR analysis, CGH,[86] and microarray analysis.[84]

Detection Methods

Once DNA from a single cell has been amplified to a detectable level, most of the mutation detection techniques currently available in diagnostic laboratories can be used for its analysis (Table 1). Mutation analyses can be broadly divided into three categories: those that are tailor made for the detection of one specific mutation, those that detect a variety of different mutations with a single protocol, and those that do not attempt to detect the mutation but infer the presence of the mutation. Techniques that fall into the first category are generally used in a diagnostic context and usually provide a rapid means of detecting common mutations. Methods in the second category are known as "scanning" methods and are usually applied to searches for mutations that have not been characterized. Scanning methodologies are particularly useful for the diagnosis of inherited disorders caused by a heterogeneous spectrum of mutations, as a single methodology can usually be applied for detection of most of the DNA sequence alterations. The third category, linkage analysis, is frequently used in the presence of a suitable pedigree, when pathological mutations are uncharacterized or when known mutations are refractory to PCR. Although such indications are encountered in couples requesting PGD, the detection of contamination and allele dropout are becoming powerful indications in their own right for the inclusion of linked markers.

Amplification Refractory Mutation System

The annealing of allele specific oligonucleotides is the basis of the amplification refractory mutation system (ARMS) technique. ARMS employs one oligonucleotide to anneal upstream of the mutation site while two other oligonucleotides each anneal exclusively to either the mutant or normal alleles. These allele-specific oligonucleotides merely serve as primers for PCR and are not detected directly. The presence or absence of a specific allele is inferred from the presence of PCR product which is only seen when primer annealing occurs. If nested PCR is used, the outer set of primers is designed to produce an amplicon containing the mutation site. Two different inner amplifications are set up from separate aliquots of the outer reaction, one containing a primer specific to the normal allele and the other a primer for the mutant allele. Amplification from only the mutant allele specific primer would result in the embryo being diagnosed as affected and excluded from transfer. Heterozygous samples would show positive amplification for both normal and mutant primer sets. As with restriction enzyme digestion-based diagnoses, the detection of both mutant and normal alleles is a safer and more informative test than the detection of the mutant allele alone. This methodology has been used for the analysis of the five most prevalent cystic fibrosis mutations in single cells[98] using a nested PCR approach.

A slight modification of this technique which has been applied clinically for the diagnosis of spinal muscular atrophy[77] allows different primers specific for mutant or normal alleles to be included in the same PCR mixture.

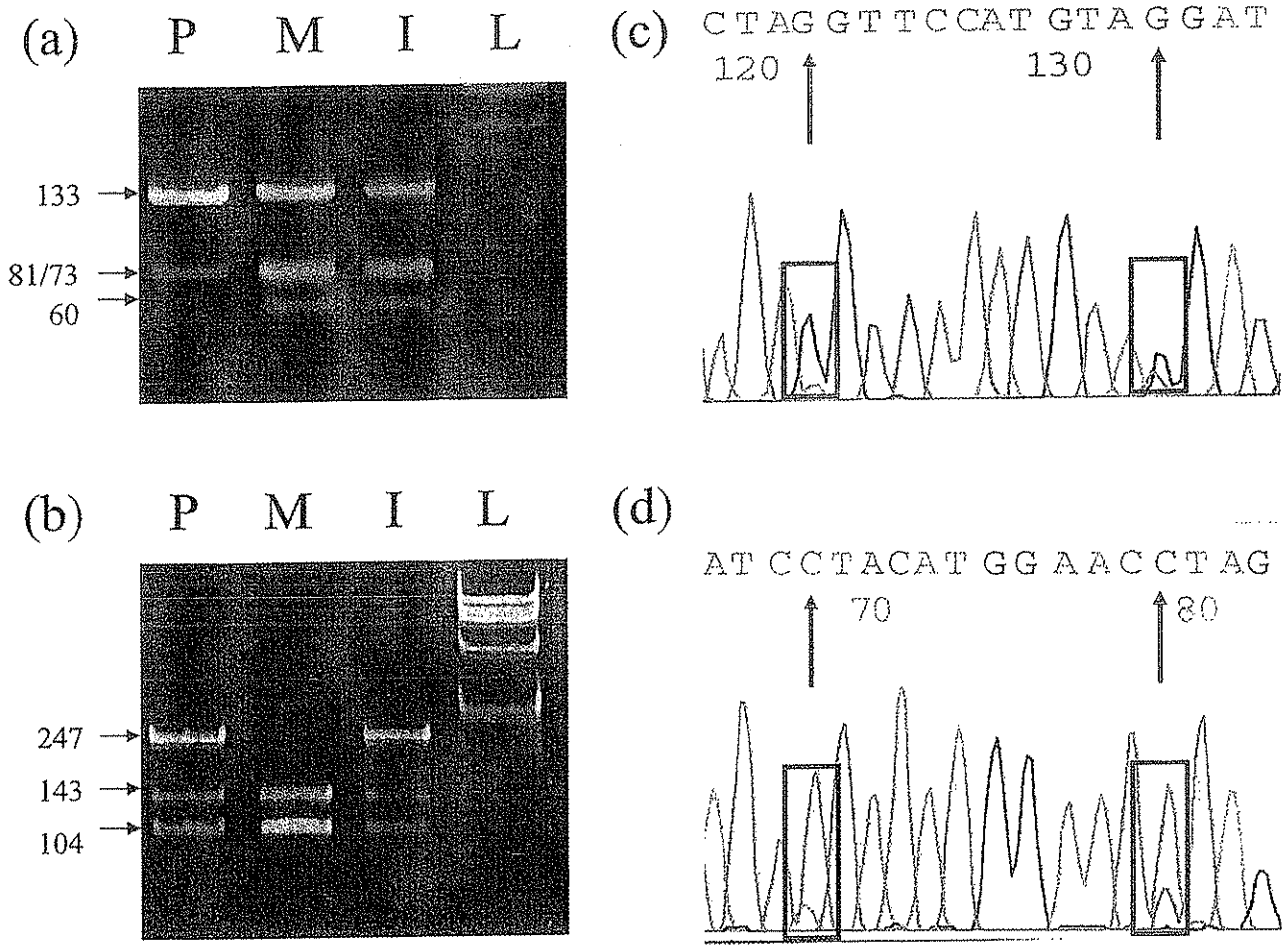

Figure 3. Restriction enzyme analysis and parallel direct sequencing of PCR products from single cells to detect two different mutations in the same PCR fragment. **a** and **b:**. Electrophoretic analysis following *Fok* I (**a**) or *Bfa* I (**b**) restriction digestion of PCR products from single lymphocytes obtained from carrier parents (each with a separate mutation in the Plakophilin 1 gene) and their affected child who is a compound heterozygote for both mutations. [M, maternal; P, paternal; I, index case (child); L, 1 kb ladder]. **a:** Maternal mutation is a T-to-G transversion which creates an additional *Fok* I cut site (*G*GATG) to generate additional digestion products (133 bp cleaved to 60 and 73 bp) in heterozygous cells (M, I). **b:** Paternal mutation is a G-to-A transition which removes an existing *Bfa* I cut site (CTA*G*) preventing complete digestion of the 247-bp product (P, I). **c** and **d:** Direct sequencing of a purified PCR product from a compound heterozygous cell (I) using big dye terminators on an ABI 310 genetic analyzer. Use of both forward (**c**) and reverse (**d**) primers clearly shows the presence of two different alleles at each mutation site (**red** and **blue boxes/arrows**). **Boxes/arrows** correspond to the location of the paternal (**red**) and maternal (**blue**) mutations respectively. Note the peak size difference between the guanine base (**black**) and the corresponding cytosine base (**blue**) at the maternal mutation site in the forward and reverse panels respectively. This observation underlines the importance of sequencing in both forward and reverse directions for single cell analysis as a precaution against preferential incorporation of different bases.

This provides rapid analysis using a single round of PCR. Normal and mutant alleles are distinguishable using automated fragment analysis following fluorescent PCR[77] or conventional electrophoresis. The latter detection method requires the design of allele specific primers with different lengths, so called allele-dependent length polymorphism (ADLP), and has been clinically applied for the preimplantation diagnosis of retinitis pigmentosa.[99]

Restriction Endonuclease Digestion

Amplification of DNA followed by restriction digestion is a common form of mutation detection in preimplantation diagnosis and has allowed single cell diagnosis of a wide variety of disorders, generally involving point mutations.[100–106] With knowledge of the DNA sequence and the exact mutation, a restriction enzyme may be selected which will cleave a normal DNA strand while a mutant strand remains undigested and the products of digestion can distinguished by electrophoresis. This is the ideal design for a clinical PGD PCR protocol. Conversely, enzymes which digest the mutant but not the normal allele can usually be found, but in such cases incomplete or failed digestion could lead to an embryo being incorrectly diagnosed as normal.[33,107–109] For such suboptimal assays the inclusion of an internal digestion control[29] could help to prevent a misdiagnosis (Figure 3).

Enzyme digestion has also been an essential component of the preimplantation diagnosis of spinal muscular atrophy (SMA), in which a causative deletion in the survival motor neuron gene (*SMN1*) prevents PCR amplification of the mutant allele, but product from a highly homologous copy gene (*SMN2*) is specifically cut by the restriction enzyme *Dra* I.[108,109] In these assays, no natu-

rally occurring restriction site exists and an artificial cut site specific for the *SMN2* is introduced during PCR using a primer mismatch, known as site specific mutagenesis (SSM), a strategy which has also been used for clinical PGD of retinitis pigmentosa.[99] An improved diagnosis for SMA uses an alternative restriction site *Hinf* I which is contained in both *SMN1* and *SMN2*. SSM was used to introduce an additional cut site in *SMN1* only to allow differentiation of the two sequences.[110] Restriction digestion is a straightforward and generally reliable method for mutation detection, but the digestion time required (between 3 and 6 hours) and the requirement for purification in some reported cases[78] can make this approach cumbersome for clinical PGD.

Heteroduplex Analysis

Heteroduplex analysis can identify a wide variety of mutations (particularly small deletions or insertions) and has been used extensively for identification of the ΔF508 mutation (a 3-bp deletion) causing cystic fibrosis. Since homozygous samples do not produce heteroduplexes, ΔF508/ΔF508 affected samples can be identified through heteroduplex formation following the addition of equivalent wild-type PCR product and absence of heteroduplex formation following addition of mutant product.[75] As well as extensive use in PGD of cystic fibrosis[22] heteroduplex analysis has also allowed diagnosis of Tay-Sachs disease[111] and was one of a series of methods used in parallel for PGD of familial adenomatous polyposis coli.[60] One potential problem with this method is the requirement for DNA of known genotype for mutation detection which could provide an opportunity for sample mix-up errors.

Single Strand Conformational Polymorphism Analysis

Single strand conformational polymorphism (SSCP) analysis is a "scanning" assay which is capable of detecting small DNA deletions and insertions and even single bp substitutions.[112] SSCP has become one of the most frequently used strategies for mutation detection and, in its simplest form, is uncomplicated and inexpensive requiring only a minimal amount of equipment. Single strands of DNA, generated by denaturing a PCR amplified sample, take on sequence specific conformations that are stabilized by intrastrand interactions. Allele-specific DNA strands frequently adopt distinct conformations which migrate at distinct rates when subjected to nondenaturing polyacrylamide gel electrophoresis. A single protocol can detect a number of genotypes so long as both mutations lie within the same amplified fragment. This may simplify the diagnosis of compound heterozygotes as such samples usually give a unique pattern of bands easily distinguished from other genotypes. Furthermore, SSCP has been performed using ethidium bromide (to detect the causative mutation in PGD of the dominant cancer syndrome familial adenomatous polyposis coli,[60] sensitive silver staining (to diagnose β-thalassemia at the single cell level),[113] or highly sensitive fluorescent PCR (to diagnose medium chain acyl CoA dehydrogenase deficiency).[76] A disadvantage of SSCP is that experimental conditions need to be carefully controlled to ensure reproducible assay sensitivity. This challenge is exacerbated by the single cell specific problems such as preferential amplification and ADO.

Denaturing Gradient Gel Electrophoresis

Denaturing gradient gel electrophoresis (DGGE) is another popular scanning method which, like SSCP, relies on physical properties of the DNA strand determined by base sequence. Mutations are detected indirectly by virtue of altered melting characteristics which affect the migration of the DNA strand as it passes through a polyacrylamide gel with increasing concentration of denaturant. The primers usually used for DNA amplification before DGGE are modified to include a stretch of approximately 40 guanine or cytosine residues (GC-clamp). These additional nucleotides significantly increase the proportion of sequence variants that can be detected in a given DNA fragment. However, under some circumstances the GC-clamp can reduce the efficiency of PCR and may actually be refractory to amplification if used at the single cell level.[114] The use of nested PCR with GC-clamped primers used only in the secondary amplification may overcome this difficulty. An advantage of DGGE over some other techniques is its ability to detect multiple mutations within the same PCR fragment. This has led to its use in clinical PGD for the detection of mutations causing β-thalassemia.[115] Like SSCP, DGGE can give banding patterns which are difficult to interpret at the single cell level.

Despite limitations in applying mutation SSCP or DGGE analysis to single cells, these methodologies can be very useful in identifying mutations in the couple before initiation of a PGD cycle. Other mutation-specific techniques or sequencing then can be used to specifically test for the parental mutations in PGD.

Sequencing

Direct sequencing is accurate, reliable and the time required to obtain results can be markedly reduced by confining the sequence analysis (post-PCR) to a smaller region of interest containing the mutation. Sequencing could be applied as a generic approach for PGD of any disease involving point mutations, small deletions, or insertions particularly when a series of mutations lie fairly close together within the same gene (as is the case for mutations in the β-globin gene resulting in thalassemia). Amplification of both parental mutation sites in the same fragment allows most ADO to be detected and prevent the transfer of affected compound heterozygous embryos.

Recently, direct sequencing was used in a PGD case involving a novel skin fragility ectodermal dysplasia syndrome to confirm restriction enzyme digestion results.[66] To attempt PGD, it was necessary to identify reliably and accurately the presence of the two different parental

mutations (which lead to a functional knockout of the plakophilin 1 gene) in a single cell assay. Fortunately, the mutations lay within 11 bp of each other making a nested PCR approach feasible for the restriction assay, whereby both mutation sites could be amplified in the same fragment during the first round of PCR. Restriction analysis was carried out using two separate digests (one for each mutation). In parallel, cycle sequencing using big dye terminators on an ABI 310 genetic analyzer (Applied Biosystems, Foster City, CA) was performed in both forward and reverse primers for each purified sample (Figure 3).

Linkage Analysis

Even when the exact mutation causing a disorder is unknown, the particular disorder may still be avoided by the detection of linked markers. Any informative polymorphism, which lies in close proximity to the disease locus, can be used as a tool to indicate the presence or absence of the mutation without its direct detection. Markers that are intragenic or situated close to the gene are preferred for this approach, as they are unlikely to be separated from the mutation by recombination during meiosis. To perform linkage analysis, a family pedigree must be obtained and DNA from family members tested to determine which polymorphic variant is inherited along with the disease phenotype. Many types of polymorphism are used for this purpose, the most commonly used are microsatellites (eg, Simple Tandem Repeats or STRs). These are highly polymorphic and consequently have the greatest probability of being informative for a given family.

Prior knowledge of the STR allele sizes of couples undergoing PGD allows the calculation of all possible zygote genotypes. Any deviation from these possibilities indicates the presence of contaminating DNA.[62,80,116,117] The polymorphic nature of STR markers also permits the detection of haploidy and uniparental disomy. For these reasons many groups involved in PGD are now attempting to incorporate polymorphic markers into their molecular diagnoses.[41,60,104] The use of tetranucleotide repeats in preference to dinucleotide repeats and the application of commercially available optimized reaction buffers should reduce the frequency of artifacts known as "stutter bands" that complicate analysis of results.[118] The preferred future method for linkage analysis may use Single Nucleotide Polymorphisms (SNPs) which are DNA alterations that occur at a frequency of approximately 1 per 1000 bp throughout the genome. Since variability at a particular locus is limited to the four deoxynucleotides, a large number of SNPs is required for reliable linkage analysis. Microarray analysis (following whole genome amplification of single cells) will be a prerequisite to using SNPs as an alternative to STRs for linkage analysis.

Linkage analysis for PGD has been used for a number of different reasons. The causative mutation may be unknown,[119] the sequence containing the mutation may be refractory to PCR,[120] or heterogeneity of the causative mutations may make linkage analysis a more cost-effective way to provide a generic test for a disorder.[121–123] In addition, detection of allele dropout (particularly relevant for dominant conditions) and contamination make linkage analysis a powerful tool in clinical PGD. Finally, linkage analysis has allowed non-disclosure testing of embryos for Huntington disease.[124] For PGD by linkage analysis, many laboratories rely on informative markers from a two-generation pedigree—frequently available in couples with previous affected pregnancies or children.

The first clinical application of linkage analysis for PGD was to identify the autosomal dominant disorder, Marfan syndrome, in which the specific mutation was unknown. Affected embryos were identified by tracing the inheritance of a dinucleotide repeat polymorphism linked to the causative fibrillin gene.[119] Since this application, linkage analysis has also been used to detect embryos carrying mutant alleles of the dystrophin gene[125] and has been combined with mutation analysis using multiplex PCR[126] or whole genome amplification.[60]

Diseases caused by the inheritance of large trinucleotide repeat expansions, such as fragile X syndrome and myotonic dystrophy, pose an additional problem for single cell analysis. In these cases the expanded allele is frequently too large to be amplified using PCR or may be subject to *in vitro* expansion producing erroneous results.[53] Consequently, inheritance of the disease allele in a tested blastomere would be inferred by the failure of PCR amplification across the expansion and the absence of the normal allele from the carrying parent. Indeed, conventional electrophoresis and later automated fragment analysis to detect non-expanded alleles was the basis for clinical preimplantation diagnosis of myotonic dystrophy[55,127] and fragile X syndrome.[128] Alternatively, linkage analysis may be used with informative markers flanking the expansion. Strategies of this kind have been successfully developed for fragile X syndrome[120,129] and myotonic dystrophy.[130] The inclusion of linked markers for the detection of allele dropout has become a standard in some laboratories and such a strategy should have a positive impact on pregnancy rates following PGD since the number of correctly diagnosed embryos available for transfer should increase as ADO is detected.[46,63]

Finally, linkage analysis can be of use in exclusion testing as a means by asymptomatic individuals who are at high risk of carrying HD can obtain antenatal genetic testing without incurring the emotional, social, and financial burdens that might result from the presymptomatic disclosure of their own carrier status.[124]

Methodologies for Future Application to Clinical PGD

Cell Recycling

Another technique, which provides cytogenetic and also molecular genetic information, is known as cell recycling.[131] Fixed single cells are subjected to sequential PCR and FISH analysis allowing the investigation of specific gene sequences as well as chromosomal copy number. This combination of information would be particularly useful for PGD cases in which patients of advanced

maternal age present with risk for having a child with a single gene disorder. Two clinical PGD cases have been reported, in which embryos free from the particular single gene disorder under test, resulted in pregnancies which miscarried and were found to be trisomy 16[132] and trisomy 22[66] respectively. In either case, an additional FISH test to rule out common chromosomal abnormalities would have been beneficial. Despite its potential, clinical application of cell recycling is not recommended at present since ADO rates are significantly higher with fixed template DNA than those observed using routine single cell protocols.[133]

Quantitative Fluorescent PCR

Quantitative fluorescent PCR (QF-PCR) assays are based on the amplification of DNA sequences unique for each chromosome pair and have been developed to establish the number of specific chromosomes present in a cell.[79] These tests amplify STR or microsatellite markers with quantitation of products. Although QF-PCR is robust and reliable and can be completed within one working day, its application at the single cell level is hampered by an unacceptably high (25%) rate of preferential amplification which results in artificially skewed ratios of PCR products and the potential for misdiagnosis of chromosomal copy number in PGD.[80] STR markers can confidently identify aneuploidy with tri-allelic trisomies in single cells but this potential has yet to be fully realized owing to a lack of highly polymorphic chromosomal markers.

Real-Time PCR

Real-time PCR allows the rate of amplicon accumulation to be measured by detection of fluorescently tagged probes at each cycle of the reaction. The use of probes directed to either wild-type or mutant sequence also allows genotyping to be performed. The technique is rapid and has the added convenience that the amplification and detection procedures are carried out in the same tube (ie, as a homogeneous assay), thereby greatly reducing the chances of laboratory contamination. For example, addition of wild-type or mutant hairpin probes (which contain a fluorophore and quencher molecule at opposite ends of the probe) allows accurate mutation analysis as PCR products accumulate in the reaction tube. As the probes anneal to target sequence, the fluorophore and quencher are separated and fluorescence can be measured. The degree of homology between probe and target determines the particular annealing temperature at which the fluorescence can be measured. Real-time PCR assays have been used very effectively to detect multiple copy Y chromosomal sequences[49] (using molecular beacon technology) and *BRCA1* sequences[134] (using LightCycler technology, Roche Diagnostics Corporation, Indianapolis, IN) in single cells and shows considerable promise for application to clinical PGD.

Denaturing High Performance Liquid Chromatography

This technique provides an efficient and inexpensive method for the rapid detection of single nucleotide mismatches and small deletions or insertions within an amplified DNA fragment without the need for fluorescence. Denaturing high performance liquid chromatography (DHPLC) exploits the differential retention of double stranded heteroduplex and homoduplex molecules, allowing the automatic comparison of PCR amplicons for variation.[135] A recent study[136] analyzing the CAG repeat region of the Huntington gene in single fibroblasts and blastomeres using this technology showed promising results in terms of amplification efficiency and ADO rates. However, aside from the markedly lower cost when compared with fluorescent PCR technology, it is difficult to see the advantages this technique can provide as fluorescent PCR becomes more readily available for routine molecular diagnostics in laboratories.

Microarrays

DNA microarrays (chips) are one of the latest and most promising tools for genetic analysis. These chips offer the possibility of simultaneously analyzing thousands of predefined DNA sequences and can be applied to DNA diagnostics, gene expression analysis, and aneuploidy detection. The most significant application has been in monitoring expression profiles to deduce genes relevant to particular disease pathologies (by comparing cDNA extracts from tissues derived from normal or disease states). Detection of aneuploidy using chip technology would work in a similar fashion to that of expression analysis.[137] Pieces of genomic DNA from specific chromosomes act as probes on the slide and a competitive hybridization process between samples from known normal karyotype and unknown occurs. Aneuploidy detection using microarrays is proving to be more difficult than expression analysis because copy number changes seen in aneuploidies are more subtle than gene expression changes which can vary by orders of magnitude.[138]

Several methodologies for mutation analysis using microarrays have also been described. One of the more common examples of this is minisequencing in which an oligonucleotide is attached to the chip by its 5′ end. Each spot on the surface of the chip can contain several million of these oligonucleotides. The oligonucleotide is complimentary to the sequence of a disease causing gene and its 3′ end terminates at the base before a known mutation site. When the surface of the chip is exposed to sample DNA with DNA polymerase and di-deoxynucleotides triphosphates, the sample DNA acts as the template for the extension. By labeling each ddNTP with a different color it is possible to determine which nucleotide was added indicating the presence or absence of the mutation. Solid-phase minisequencing following whole genome amplification by PEP correctly genotyped single cells at 96% of the nucleotide positions analzyed.[84] Current drawbacks to using microarray technology in PGD include high cost, poor reproducibility, complex and

Table 5. Quality Control/Quality Assurance for Single Cell PCR

Process	Measures
Routine/general QC	Comprehensive training/protocols (esp. contamination control)
	Avoid specimen mix-up (multiple samples/embryos per patient)
	Overlap batches of tested and untested reagents
	Test all reagents prior to a clinical case
	Check temperatures of water-baths/thermal cyclers, etc
	Pipette calibration
	External quality assessment (unavailable at present)
Ensure appropriate testing	Medical genetics consultation recommended
	Verification of diagnosis (documentation or laboratory re-test)
	Apply PGD test offered to DNA/single cells from the couple
	Karyotype couple to exclude chromosomal abnormality?
Assay development	Minimum number of single cells analyzed for assay development
	Use heterozygous single cells to establish ADO/amplification rates
	Blastomere analysis for assay development
	Analyze DNA from −/−, +/− and +/+ sources
	Optimize primer design (particularly first round of nested PCR)
	Perform "dummy-runs" in simulated case conditions
Clinical assay	Selection of mononucleate blastomeres only for analysis
	Observe cell transfer to reaction tube
	Use of check gel to avoid post-PCR processing of failed samples
	Use of positive and multiple negative controls per clinical assay
	Contamination (observe precautions described in Table 3)
	Allele dropout (observe precautions described in Table 4)
	Minimize turn-around-time (for timely embryo selection/transfer)
Mutation detection	Design PCR such that normal allele is cut into new product sizes
	Use of internal controls for restriction enzyme digestion
	Purify PCR product prior to restriction digestion (if necessary)
	Establish cut-off values for failed amplifications/contamination in fluorescent PCR
	Sequence using forward and reverse primers
Documentation	Labeling ± color coding of tubes
	Worksheet to contain all tube labels, gel loading sequence, etc.
	Diagnostic laboratory supervisor/director sign off for all cases
	Witness in IVF laboratory for embryo selection and transfer
Misdiagnosis rates/ADO rates	Assess single blastomeres from non-transferred embryos
	Confirm PGD result by amniocentesis/CVS/cord blood

lengthy data analysis, and the absolute requirement for some form of whole genome amplification.

Quality Control and Quality Assurance for Single Cell PCR

Reliability and accuracy in any molecular genetics diagnostics laboratory rely on stringent quality control (QC) and quality assurance (QA) measures, many of which are specific to PCR.[139] Such routine QC and QA measures would include appropriate assay validation, participation in proficiency testing surveys, testing of reagents before a clinical case, incorporation of measures to prevent and detect sample mix-up or contamination, routine equipment maintenance, and laboratory accreditation. The costs of these standard measures are generally absorbed within a general quality assurance plan in larger reference diagnostic laboratories. To ensure the highest standards of analytic reliability and accuracy for single cell analyses, additional measures are required (Table 5). The combination of general and single cell specific QC/QA costs could be prohibitive in IVF laboratories providing single cell diagnostics.

For example, primer design is critical when working with only 7 pg of DNA. In nested PCR, the outer primers are more critical than the inner primers and will have a significant effect on ADO rates if suboptimal. In view of the time-constraints for clinical PGD, smaller PCR products can help to reduce timings for electrophoretic separation and fragment analysis and even accentuate small differences in allele sizes on conventional gels. Optimization of the reaction with respect to magnesium and primer concentration, annealing temperatures and so on can be achieved using small amounts of informative DNA (20 to 50 ng) before using readily available single cells (such as lymphocytes, buccals cells, or fibroblasts). It is imperative to optimize the assay using such single cells since amplification rates in blastomeres are frequently lower and more variable. The minimum number of single cells for such validation studies has yet to be standardized. However, it is not unreasonable to conduct a series of between 5 and 10 consecutive experiments with at least 10 to 20 single cells per experiment to establish amplification efficiency and contamination rates with any degree of confidence. Furthermore, ADO rates can be established using heterozygous cells which can be obtained from commercial sources (as lymphoblast or fibroblast cell lines) or fresh from known carriers and prospective PGD patients (lymphocytes or buccal cells). The use of embryonic blastomeres as part of the assay development before clinical test implementation is controversial since a given mutation will be absent in most embryos

generated by routine IVF (donated to research in excess of clinical need). Indeed, the majority of such results will be uninformative with respect to allele dropout rates. However, the use of non-transferred embryos (surplus in a clinical PGD case) is recommended to obtain misdiagnosis rates on embryos informative for the particular assay. Although a minimum number of single cells for assay validation has yet to be determined, development studies can be expensive, particularly in view of the need for large numbers of contamination controls. With a shortage of generic tests applicable at the single cell level for single gene disorders exhibiting molecular heterogeneity, the high cost of custom assay development has, so far, been a limiting factor in the uptake of PGD. Furthermore, a single PGD treatment involving 10 embryos could create up to 45 samples (depending on the number of blastomeres and appropriate controls analyzed) making the cost of reagents and personnel time significantly different compared with analysis of a single blood sample. As high-throughput technologies become widespread, however, the cost of molecular testing to the patient should not be prohibitive. Indeed, in the United States, the diagnostic test itself probably accounts for less than 10% of the total cost of the PGD treatment, the IVF procedures accounting for most of the cost.

In view of the cost of PGD treatment and the unique nature and origin of the test material, the additional cost and inconvenience to the patient of pre-cycle screening to ensure appropriateness of testing is justified. Before commencing a PGD cycle, it is vital to verify the DNA diagnosis using peripheral blood from the couple. Furthermore, it is prudent to apply the specific PGD test to DNA or single cells from the particular couple to discover any unexpected test results which could render future blastomere results questionable (for example, a polymorphism which may exist under a primer used in the single cell assay but not in the routine laboratory assay).

A number of QC processes apply to the single cell PCR procedure itself relating to amplification failure, contamination (Table 3), and allele dropout (Table 4). With respect to amplification failure, intrinsic problems relating to the biopsied material can be reduced by selecting only mononucleate blastomeres for analysis and using a check gel (when appropriate) to avoid time-consuming and costly post-PCR processing of failed samples. This latter measure is particularly important in view of the high number of samples expected to have no amplification (ie, wash blank controls). The number of blanks to include in assay development and clinical cases presents something of a dilemma in view of the calculation that 300 negative blanks are required to ensure that the contamination rate is less than 1%. A two-stage testing procedure has been suggested to maintain this low contamination rate. Before clinical implementation, a large series of blanks (eg, 100) should be run. After this, smaller series should be run periodically.[63] Specific QC and QA measures taken for mutation detection procedures have been discussed in previous sections of this review and are listed in Table 5.

Finally, the documentation and hand-over procedures in clinical PGD must be stringent to avoid sample mix-up at any stage of the process. Such procedures are critical for the reporting of any genetic test result. Fortunately, some of the more recent technologies (such as real-time PCR) should make it possible to avoid transfer of material between tubes since amplification and mutation detection takes place in the same tube. Regardless of this possibility, it is recommended that critical procedures (eg, transfer of embryos between dishes) be witnessed by a second person and a rigorous protocol for labeling tubes and loading gels be implemented.

One QA measure, conspicuous by its absence in clinical PGD at present, is external quality assessment (EQA) or proficiency testing. If satellite PGD (in which IVF laboratories collect embryonic material for analysis at distant diagnostic laboratories) is to become more accepted, EQA is essential to maintain the highest standards of patient care. The different mutation detection strategies used to diagnose the same disorders (as shown in Table 1) demonstrate the lack of consensus and standardization in PGD. An analogous area of genetic testing is PCR screening for Y chromosomal microdeletions in the work-up for male infertility in which an EQA project is providing laboratories worldwide with overall misdiagnosis rates and an individual performance rating.[140] Organizing a similar scheme for PGD is essential but represents an enormous challenge which may ultimately only be met under the auspices of such organizations as the ESHRE PGD consortium[1,16] or the International Working Group on Preimplantation Genetics.[141]

Ethical, Legal and Social Issues Relating to PGD

Considerable differences in the regulatory oversight of PGD exists among countries, ranging from total bans on any embryo manipulation to the almost complete absence of any regulations or authority.[142] The high cost of practice, low pregnancy rate,[143] problems with patient access, and insurance coverage appear to be the biggest drawbacks to universal acceptance in societal terms. Ethical discussions considering the moral status of the human embryo[144] and what constitutes severe genetic disease have been debated elsewhere[145,146] but such discussions are clearly outside of the purview of this methodological review. Somewhat reassuring for those centers currently offering PGD is the acknowledgment from professional organizations that PGD can now be considered a "standard of care" rather than an experimental treatment.[147]

Conclusions

Robust and reliable single cell PCR diagnoses require optimization of reaction conditions and appropriate mutation detection strategies. For this to be achieved, one must fully appreciate the difficulties of amplifying DNA from a single cell. Once the limitations of a single cell have been overcome, using some form of DNA amplification, the mutation detection methods available in the

molecular genetics armory are all applicable with only minor modifications.

The use of informative polymorphisms, which can provide confirmatory results to mutation analysis, identify contamination and increase the detection rate of ADO, will increase the reliability and accuracy of many of the diagnostic strategies already reported. For single cell applications, fluorescent PCR will likely replace conventional PCR strategies in view of its speed, throughput, and sensitivity, all of which could help to reduce the cost of each diagnostic test. Increasing amounts of chromosomal information from single cells will also be obtained using PCR-based techniques such as improved methods of quantitative fluorescent PCR and whole genome amplification in combination with comparative genomic hybridization or microarray technology. Whatever the difficulties faced by single cell diagnosis, the growing patient demand for PGD will continue to drive research into the application of further strategies for the diagnosis of an increasing variety of inherited diseases.

As can be seen from Table 1, a large number of assays have been developed over the past decade to detect a variety of disorders. Development of any single cell assay can be costly and time-consuming and the development of assays for couples with unique mutations is a tribute to the dedication of researchers in the field of preimplantation genetics. However, the focus of the next decade should be to develop robust single cell assays with an emphasis on making such tests generic (for example, using linkage analysis with STRs or SNPs) to use limited resources cost-effectively to help more couples. Each of the various mutation detection methods and PCR strategies described above is associated with a different turnaround time (for example, real-time PCR requires less time than nested PCR followed by restriction digestion). The improvement of embryo culture medium supporting embryo development to the blastocyst stage now provides up to 3 days for analysis, more than enough time for any of the methods described above. Expansion of the analytic window has also made possible the geographical separation of IVF center and diagnostic laboratory, although significant logistics issues remain.

In conclusion, PGD testing is largely unregulated by any accrediting agency at present. The introduction of standardization, proficiency testing, and external quality assessment procedures among centers offering PGD (whether on-site or at a satellite laboratory) is in accordance with other forms of molecular testing and would ensure the highest quality of care for all patients.

References

1. ESHRE preimplantation genetic diagnosis (PGD) consortium: data collection II. Hum Reprod 2000, 15:2673–2683
2. Handyside AH, Kontogianni EH, Hardy K, Winston RM: Pregnancies from biopsied human preimplantation embryos sexed by Y-specific DNA amplification. Nature 1990, 344:768–770
3. Avner R, Reubinoff BE, Simon A, Zentner BS, Friedmann A, Mitrani Rosenbaum S, Laufer N: Management of rhesus isoimmunization by preimplantation genetic diagnosis. Mol Hum Reprod 1996, 2:60–62
4. Chong SS, Kristjansson K, Cota J, Handyside AH, Hughes MR: Preimplantation prevention of X-linked disease: reliable and rapid sex determination of single human cells by restriction analysis of simultaneously amplified ZFX and ZFY sequences. Hum Mol Genet 1993, 2:1187–1191
5. Nakahori Y, Hamano K, Iwaya M, Nakagome Y: Sex identification by polymerase chain reaction using X-Y homologous primer. Am J Med Genet 1991, 39:472–473
6. Levinson G, Keyvanfar K, Wu JC, Fugger EF, Fields RA, Harton GL, Palmer FT, Sisson ME, Starr KM, Dennison-Lagos L, Calvo L, Sherins RJ, Bick D, Schulman JD, Black SH: DNA-based X-enriched sperm separation as an adjunct to preimplantation genetic testing for the prevention of X-linked disease: Hum Reprod 1995, 10:979–982
7. Hashiba T, Sueoka K, Kuroshima M, Asada H, Kuji N, Yoshimura Y: Accurate multiplex polymerase chain reaction assay for gender determination from a single cell. Gynecol Obstet Invest 2000, 49: 217–220
8. Griffin DK, Wilton LJ, Handyside AH, Atkinson GH, Winston RM, Delhanty JD: Diagnosis of sex in preimplantation embryos by fluorescent in situ hybridisation. Br Med J 1993, 306:1382
9. Grifo JA, Tang YX, Munne S, Alikani M, Cohen J, Rosenwaks Z : Healthy deliveries from biopsied human embryos. Hum Reprod 1994, 9:912–916
10. Liu J, Lissens W, Van Broeckhoven C, Lofgren A, Camus M, Liebaers I, Van Steirteghem A: Normal pregnancy after preimplantation DNA diagnosis of a dystrophin gene deletion. Prenat. Diagn 1995, 15:351–358
11. Hussey ND, Donggui H, Froiland DA, Hussey DJ, Haan EA, Matthews CD, Craig JE: Analysis of five Duchenne muscular dystrophy exons and gender determination using conventional duplex polymerase chain reaction on single cells. Mol Hum Reprod 1999, 5:1089–1094
12. Ray PF, Vekemans M, Munnich A: Single cell multiplex PCR amplification of five dystrophin gene exons combined with gender determination. Mol Hum Reprod 2001, 7:489–494
13. Harper JC, Thornhill AR: Embryo biopsy. Preimplantation Genetic Diagnosis. Edited by Harper JC, Delahanty JDA, Handyside AH. Chicester, UK, John Wiley and Sons, 2001, pp 141–163
14. Verlinsky Y, Ginsberg N, Lifchez A, Valle J, Moise J, Strom CM: Analysis of the first polar body: preconception genetic diagnosis. Hum Reprod 1990, 5:826–829
15. Verlinsky Y, Rechitsky S, Cieslak J, Ivakhnenko V, Wolf G, Lifchez A, Kaplan B, Moise J, Walle J, White M, Ginsberg N, Strom C, Kuliev A: Preimplantation diagnosis of single gene disorders by two-step oocyte genetic analysis using first and second polar body. Biochem Mol Med 1997, 62:182–187
16. ESHRE PGD Consortium Steering Committee: ESHRE Preimplantation Genetic Diagnosis (PGD) Consortium: preliminary assessment of data from January 1997 to September 1998. Hum Reprod 1999, 14:3138–3148
17. Hardy K, Martin KL, Leese HJ, Winston RML, Handyside AH: Human preimplantation development in vitro is not adversely affected by biopsy at the 8-cell stage. Hum Reprod 1990, 5:708–714
18. Dokras A, Sargent IL, Ross C, Gardner RL, Barlow DH: Trophectoderm biopsy in human blastocysts. Hum Reprod 1990, 5:821–825
19. Pickering SJ, Muggleton-Harris AL: Reliability and accuracy of polymerase chain reaction amplification of two unique target sequences from biopsies of cleavage-stage and blastocyst-stage human embryos. Hum Reprod 1995, 10:1021–1029
20. Veiga A, Sandalinas M, Benkhalifa M, Boada M, Carrera M, Santalo J, Barri PN, Menezo Y: Laser blastocyst biopsy for preimplantation genetic diagnosis in the human. Zygote 1997, 5:351–354
21. Cui KH, Matthews CD: Nuclear structural conditions and PCR amplification in human preimplantation diagnosis. Mol Hum Reprod 1996, 2:63–71
22. Ray PF, Ao A, Taylor DM, Winston RML, Handyside AH: Assessment of the reliability of single blastomere analysis for preimplantation diagnosis of the ΔF508 deletion causing cystic fibrosis in clinical practice. Prenat Diagn 1998, 18:1402–1412
23. Avner R, Laufer N, Safran A, Kerem BS, Friedmann A, Mitrani Rosenbaum S: Preimplantation diagnosis of cystic fibrosis by simultaneous detection of the W1282X and ΔF508 mutations. Hum. Reprod 1994, 9:1676–1680
24. Sermon K, Lissens W, Nagy ZP, Van Steirteghem A, Liebaers I: Simultaneous amplification of the two most frequent mutations of

infantile Tay-Sachs disease in single blastomeres. Hum Reprod 1995, 10:2214–2217
25. Gitlin SA, Lanzendorf SE, Gibbons WE: Polymerase chain reaction amplification specificity: incidence of allele dropout using different DNA preparation methods for heterozygous single cells. J Assist Reprod Genet 1996, 13:107–111
26. Kontogianni EH, Griffin DK, Handyside AH: Identifying the sex of human preimplantation embryos in X-linked disease: amplification efficiency of a Y-specific alphoid repeat from single blastomeres with two lysis protocols. J Assist Reprod Genet 1996, 13:125–132
27. Ray PF, Handyside AH: Increasing the denaturation temperature during the first cycles of amplification reduces allele dropout from single cells for preimplantation genetic diagnosis. Mol Hum Reprod 1996, 2:213–218
28. El-Hashemite N, Delhanty JDA: A technique for eliminating allele specific amplification failure during DNA amplification of heterozygous cells for preimplantation diagnosis. Mol Hum Reprod 1997, 3:975–978
29. Thornhill AR, McGrath JA, Braude P, Eady R, Handyside AH: A comparison of different lysis buffers to assess allele dropout from single cells for preimplantation genetic diagnosis. Prenat Diagn 2001, 21:490–497
30. Kwok S, Higuchi R: Avoiding false positives with PCR. Nature 1989, 339:237–238
31. Zhu YS, Isaacs ST, Cimino CD, Hearst JE: The use of exonuclease III for polymerase chain reaction sterilization. Nucleic Acids Res 1991, 19:2511
32. Sermon K, Goossens V, Seneca S, Lissens W, De Vos A, Vandervorst M, Van Steirteghem A, Liebaers I: Preimplantation diagnosis for Huntington's disease (HD): clinical application and analysis of the HD expansion in affected embryos. Prenat Diagn 1998, 18: 1427–1436
33. Sermon K, Henderix P, Lissens W, De Vos A, Vandervorst M, Vanderfaeillie A, Vamos E, Van Steirteghem A, Liebaers I: Preimplantation genetic diagnosis for medium-chain acyl-CoA dehydrogenase (MCAD) deficiency. Mol Hum Reprod 2000, 6:1165–1168
34. De Rycke M, Van de Velde H, Sermon K, Lissens W, De Vos A, Vandervorst M, Vanderfaeillie A, Van Steirteghem A, Liebaers I: Preimplantation genetic diagnosis for sickle-cell anemia and for beta-thalassemia. Prenat Diagn 2001, 21:214–222
35. Prince AM, Andrus L: PCR: how to kill unwanted DNA. Biotechniques 1992, 12:358–360
36. Longo MC, Berninger MS, Hartley JL: Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions. Gene 1990, 93:125–128
37. Cimino GD, Metchette KC, Tessman JW, Hearst JE, Isaacs ST: Post-PCR sterilization: a method to control carryover contamination for the polymerase chain reaction. Nucleic Acids Res 1991, 19:99–107
38. Liu J, Lissens W, Silber SJ, Devroey P, Liebaers I, Van Steirteghem A: Birth after preimplantation diagnosis of the cystic fibrosis delta F508 mutation by polymerase chain reaction in human embryos resulting from intracytoplasmic sperm injection with epididymal sperm. JAMA 1994, 272:1858–1860
39. Ray PF, Winston RML, Handyside AH: Single cell analysis for diagnosis of cystic fibrosis and Lesch-Nyhan syndrome in human embryos before implantation. Miami Bio/Technol 1994, 5:46 (Abstract)
40. Findlay I, Ray P, Quirke P, Rutherford A, Lilford R: Allelic drop-out and preferential amplification in single cells and human blastomeres: implications for preimplantation diagnosis of sex and cystic fibrosis. Hum Reprod 1995, 10:1609–1618
41. Rechitsky S, Strom C, Verlinsky O, Amet T, Ivakhnenko V, Kukharenko V, Kuliev A, Verlinsky Y: Allele dropout in polar bodies and blastomeres. J Assist Reprod Genet 1998, 15:253–257
42. Hahn S, Garvin AM, Di Naro E, Holzgreve W: Allele drop-out can occur in alleles differing by a single nucleotide and is not alleviated by preamplification or minor template increments. Genet Test 1998, 2:351–355
43. Navidi W, Arnheim N: Using PCR in preimplantation genetic disease diagnosis. Hum Reprod 1991, 6:836–849
44. Ao A, Ray P, Harper J, Lesko J, Paraschos T, Atkinson G, Soussis I, Taylor D, Handyside A, Hughes M, Winston RM: Clinical experience with preimplantation genetic diagnosis of cystic fibrosis (delta F508). Prenat Diagn 1996, 16:137–142
45. De Vos A, Sermon K, Van de Velde H, Joris H, Vandervorst M, Lissens W, Mortier G, DeSutter P, Lofgren A, Van Broeckhoven C, Liebaers I, Van Steirteghem A: Pregnancy after preimplantation genetic diagnosis for Charcot-Marie-Tooth disease type 1A. Mol Hum Reprod 1998, 4:978–984
46. Verlinsky Y, Kuliev A: An Atlas of Preimplantation Genetic Diagnosis. New York, Parthenon, 2000
47. Findlay I, Matthews P, Quirke P: Multiple genetic diagnoses from single cells using multiplex PCR: reliability and allele dropout. Prenat Diagn 1998, 18:1413–1421
48. Pickering SJ, McConnell JM, Johnson MH, Braude PR: Reliability of detection by polymerase chain reaction of the sickle cell-containing region of the beta-globin gene in single human blastomeres. Hum Reprod 1992, 7:630–636
49. Pierce KE, Rice JE, Sanchez JA, Brenner C, Wangh LJ: Real-time PCR using molecular beacons for accurate detection of the Y chromosome in single human blastomeres. Mol Hum Reprod 2000, 6:1155–1164
50. Kuo HC, Mackie Ogilvie C, Handyside AH: Chromosomal mosaicism in cleavage-stage human embryos and the accuracy of single-cell genetic analysis. J Assist Reprod Genet 1996, 15:276–280
51. Harper JC, Coonen E, Handyside AH, Winston RML, Hopman AHN, Delhanty JDA: Mosaicism of autosomes and sex chromosomes in morphologically normal, monospermic preimplantation human embryos. Prenat Diagn 1995, 15:41–49
52. Wells D, Sherlock JK: Strategies for preimplantation genetic diagnosis of single gene disorders by DNA amplification. Prenat Diagn 1998, 18:1389–1401
53. Daniels R, Holding C, Kontogianni E, Monk M: Single-cell analysis of unstable genes. J Assist Reprod Genet 1996, 13:163–169
54. Drury KC, Liu MC, Kipersztok S, Williams RS: Reduced incidence of allele dropout using SYBR Green I and PCR identification of X and Y amelogenin gene in preimplantation genetic diagnosis (PGD). Fertil Steril 1997, 68:S151–S152 (Abstract)
55. Sermon K, De Vos A, Van de Velde H, Seneca S, Lissens W, Joris H, Vandervorst M, Van Steirteghem A, Liebaers I: Fluorescent PCR and automated fragment analysis for the clinical application of preimplantation genetic diagnosis of myotonic dystrophy (Steinert's disease). Mol Hum Reprod 1998, 4:791–796
56. Eldadah ZA, Grifo JA, Dietz HC: Marfan syndrome as a paradigm for transcript-targeted preimplantation diagnosis of heterozygous mutations. Nat Med 1995, 1:798–803
57. Daniels R, Adjaye J, Bolton V, Monk M: Detection of a novel splice variant of the hypoxanthine-guanine phosphoribosyl transferase gene in human oocytes and preimplantation embryos: implications for a RT-PCR-based preimplantation diagnosis of Lesch-Nyhan syndrome. Mol Hum Reprod 1998, 4:785–789
58. Tsai YH: Cost-effective screening methods for various single gene defects in single cells using high magnesium and total ionic strength and restriction enzymes. Prenat Diagn 2000, 20:979–985
59. Schulze E, Bettendorf M, Maser-Gluth C, Decker M, Schwabe U: Allele dropout using PCR-based diagnosis for the splicing mutation in intron 2 of the CYP21B gene: successful amplification with a *Taq/Pwo*-polymerase mixture. Endocr Res 1998, 24:637–641
60. Ao A, Wells D, Handyside AH, Winston RM, Delhanty JD: Preimplantation genetic diagnosis of inherited cancer: familial adenomatous polyposis coli. J Assist Reprod Genet 1998, 15:140–144
61. Rechitsky S, Strom C, Verlinsky O, Amet T, Ivakhnenko V, Kukharenko V, Kuliev A, Verlinsky Y: Accuracy of preimplantation diagnosis of single-gene disorders by polar body analysis of oocytes. J Assist Reprod Genet 1999, 16:192–198
62. Findlay I, Urquhart A, Quirke P, Sullivan K, Rutherford A, Lilford R: Simultaneous DNA "fingerprinting," diagnosis of sex and single-gene defect status from single cells. Mol Hum Reprod 1995, 10: 1005–1013
63. Lewis CM, Pinel T, Whittaker JC, Handyside AH: Controlling misdiagnosis errors in preimplantation genetic diagnosis: a comprehensive model encompassing extrinsic and intrinsic sources of error. Hum Reprod 2001, 16:43–50
64. Ray PF, Kaeda JS, Bingham J, Roberts I, Handyside AH: Preimplantation genetic diagnosis of beta-thalassemia major. Lancet 1996, 347:1696
65. El-Hashemite N, Wells D, Delhanty JD: Preimplantation genetic diagnosis of beta-thalassaemia. Lancet 1996, 348:620–621

66. Thornhill AR, Pickering SJ, Whittock N, Caller J, Andritsos V, Handyside AH, Braude PR, Eady RA, McGrath J: Preimplantation genetic diagnosis of compound heterozygous mutations leading to ablation of Plakophilin-1 (PKP1) and resulting in skin fragility ectodermal dysplasia syndrome. Prenat Diagn 2000, 20:1055–1062
67. Van de Velde H, De Vos A, Sermon K, Staessen C, De Rycke M, Van Assche E, Lissens W, Vandervorst M, Van Ranst H, Liebaers I, Van Steirteghem A: Embryo implantation after biopsy of one or two cells from cleavage-stage embryos with a view to preimplantation genetic diagnosis. Prenat Diagn 2000, 20:1030–1037
68. Sago H, Kim HS, Goldberg JD, Chung JH, Pedersen RA, Lebo RV: Dual blastomere analysis improves reliability of preimplantation trembler mouse diagnosis. Hum Genet 1997, 101:223–228
69. Kim HS, Klimanskaya IV, Damsky CK, Pedersen RA, Lebo RV: Preimplantation diagnosis of the beta-1 integrin knockout mutation as a model for aneuploid gene testing. Hum Genet 1999, 105:480–488
70. Garvin A, Holzgreve W, Hahn S: Highly accurate analysis of heterozygous loci by single cell PCR. Nucleic Acids Res 1998, 26: 3468–3472
71. Li HH, Gyllensten UB, Cui XF, Saiki RK, Erlich HA, Arnheim N: Amplification and analysis of DNA sequences in single human sperm and diploid cells. Nature 1988, 335:414–417
72. Holding C, Monk M: Diagnosis of beta-thalassemia by DNA amplification in single blastomeres from mouse preimplantation embryos. Lancet 1989, 2:532–535
73. Moutou C, Viville S: Improvement of preimplantation genetic diagnosis (PGD) for the cystic fibrosis mutation delta F508 by fluorescent polymerase chain reaction. Prenat Diagn 1999, 19:1248–1250
74. Hattori M, Yoshioka K, Sakaki Y: High-sensitive fluorescent DNA sequencing and its application for detection and mass-screening of point mutations. Electrophoresis 1992, 13:560–565
75. Handyside AH, Lesko JG, Tarin JJ, Winston RM, Hughes MR: Birth of a normal girl after in vitro fertilization and preimplantation diagnostic testing for cystic fibrosis. N Engl J Med 1992, 327:905–909
76. Ioulianos A, Wells D, Harper JC, Delhanty JD: A successful strategy for preimplantation diagnosis of medium-chain acyl-CoA dehydrogenase (MCAD) deficiency. Prenat Diagn 2000, 20:593–598
77. Moutou C, Gardes N, Rongieres C, Ohl J, Bettahar-Lebugle K, Wittemer C, Gerlinger P, Viville S: Allele-specific amplification for preimplantation genetic diagnosis (PGD) of spinal muscular atrophy. Prenat Diagn 2001, 21:498–503
78. Van de Velde H, Sermon K, De Vos A, Lissens W, Joris H, Vandervorst M, Van Steirteghem A, Liebaers I: Fluorescent PCR and automated fragment analysis in preimplantation genetic diagnosis for 21-hydroxylase deficiency in congenital adrenal hyperplasia. Mol Hum Reprod 1999, 5:691–696
79. Pertl B, Weitgasser U, Kopp S, Kroisel PM, Sherlock J, Adinolfi M: Rapid detection of trisomies 21 and 18 and sexing by quantitative fluorescent multiplex PCR. Hum Genet 1996, 98:55–59
80. Sherlock J, Cirigliano V, Petrou M, Tutschek B, Adinolfi M: Assessment of quantitative fluorescent multiplex PCR performed on single cells. Ann Hum Genet 1998, 62:9–23
81. Blake D, Tan SL, Ao A: Assessment of multiplex fluorescent PCR for screening single cells for trisomy 21 and single gene defects. Mol Hum Reprod 1999, 5:1166–1175
82. Eggerding FA, Iovannisci DM, Brinson E, Grossman P, Winn-Deen ES: Fluorescence-based oligonucleotide ligation assay for analysis of cystic fibrosis transmembrane conductance regulator gene mutations. Hum Mutat 1995, 5:153–165
83. Kuliev A, Rechitsky S, Verlinsky O, Ivakhnenko V, Evsikov S, Wolf G, Angastiniotis M, Georghiou D, Kukharenko V, Strom C, Verlinsky Y: Preimplantation diagnosis of thalassemias. J Assist Reprod Genet 1998, 15:219–225
84. Paunio T, Reima I, Syvanen AC: Preimplantation diagnosis by whole-genome amplification, PCR amplification, and solid-phase minisequencing of blastomere DNA. Clin Chem 1996, 42:1382–1390
85. Zhang L, Cui X, Schmitt K, Hubert R, Navidi W, Arnheim N: Whole genome amplification form a single cell: implications for genetic-analysis. Proc Natl Acad Sci USA 1992, 89:5847–5851
86. Wells D, Sherlock JK, Handyside AH, Delhanty JD: Detailed chromosomal and molecular genetic analysis of single cells by whole genome amplification and comparative genomic hybridisation. Nucleic Acids Res 1999, 27:1214–1218
87. Gibbons WE, Gitlin SA, Lanzendorf SE: Strategies to respond to polymerase chain reaction deoxyribonucleic acid amplification failure in a preimplantation genetic diagnosis program. Am J Obstet Gynecol 1995, 172:1088–1095
88. Schaaff F, Wedemann H, Schwinger E: Analysis of sex and delta F508 in single amniocytes using primer extension preamplification. Hum Genet 1996, 98:158–161
89. Xu K, Tang Y, Grifo JA, Rosenwaks Z, Cohen J: Primer extension preamplification for detection of multiple genetic loci from single human blastomeres. Hum Reprod 1993, 8:2206–2210
90. Snabes MC, Chong SS, Subramanian SB, Kristjansson K, DiSepio D, Hughes MR: Preimplantation single-cell analysis of multiple genetic loci by whole-genome amplification. Proc Natl Acad Sci USA 1994, 91:6181–6185
91. Kristjansson K, Chong SS, Van den Veyver IB, Subramanian S, Snabes MC, Hughes MR: Preimplantation single cell analyses of dystrophin gene deletions using whole genome amplification. Nat Genet 1994, 6:19–23
92. Sermon K, Lissens W, Joris H, Van Steirteghem A, Liebaers I: Adaptation of the primer extension preamplification (PEP) reaction for preimplantation diagnosis: single blastomere analysis using short PEP protocols. Mol Hum Reprod 1996, 2:209–212
93. Telenius H, Carter NP, Bebb CE, Nordenskjold M, Ponder BA, Tunnacliffe A: Degenerate oligonucleotide-primed PCR: general amplification of target DNA by a single degenerate primer. Genomics 1992, 13:718–725
94. Wells D, Delhanty JD: Comprehensive chromosomal analysis of human preimplantation embryos using whole genome amplification and single cell comparative genomic hybridization. Mol Hum Reprod 2000, 6:1055–1062
95. Voullaire L, Slater H, Williamson R, Wilton L: Chromosome analysis of blastomeres from human embryos by using comparative genomic hybridization. Hum Genet 2000, 106:210–217
96. Wilton L, Williamson R, McBain J, Edgar D, Voullaire L: Birth of a healthy infant after preimplantation confirmation of euploidy by comparative genomic hybridization. N Engl J Med 2001, 21:1537–1541
97. Focault F, Praz F, Jaulin C, Amor-Gueret M: Experimental limits of PCR analysis of $(CA)_n$ repeat alterations. Trends Genet 1996, 12: 450–452
98. Scobie G, Woodroffe B, Fishel S, Kalsheker N: Identification of the five most common cystic fibrosis mutations in single cells using a rapid and specific differential amplification system. Mol Hum Reprod 1996, 2:203–207
99. Strom CM, Rechitsky S, Wolf G, Cieslak J, Kuliev A, Verlinsky Y: Preimplantation diagnosis of autosomal dominant retinitis pigmentosum using two simultaneous single cell assays for a point mutation in the rhodopsin gene. Mol Hum Reprod 1998, 4:351–355
100. Cserhalmi-Friedman PB, Tang Y, Adler A, Krey L, Grifo JA, Christiano AM: Preimplantation genetic diagnosis in two families at risk for recurrence of Herlitz junctional epidermolysis bullosa. Exp Dermatol 2000, 9:290–297
101. Ray PF, Harper JC, Ao A, Taylor DM, Winston RM, Hughes M, Handyside AH: Successful preimplantation genetic diagnosis for sex -linked Lesch-Nyhan syndrome using specific diagnosis. Prenat Diagn 1999, 19:1237–1241
102. Xu K, Shi ZM, Veeck LL, Hughes MR, Rosenwaks Z: First unaffected pregnancy using preimplantation genetic diagnosis for sickle cell anemia. JAMA 281:1701–1706
103. Verlinsky Y, Rechitsky S, Schoolcraft W, Strom C, Kuliev A: Preimplantation diagnosis for Fanconi anemia combined with HLA matching. JAMA 2001, 285:3130–3133
104. Ray PF, Gigarel N, Bonnefont JP, Attie T, Hamamah S, Frydman N, Vekemans M, Frydman R, Munnich A: First specific preimplantation genetic diagnosis for ornithine transcarbamylase deficiency. Prenat Diagn 2000, 20:1048–1054
105. De Vos A, Sermon K, Van de Velde H, Joris H, Vandervorst M, Lissens W, De Paepe A, Liebaers I, Van Steirteghem A: Two pregnancies after preimplantation genetic diagnosis for osteogenesis imperfecta type I and type IV. Hum Genet 2000, 106:605–613
106. Goossens V, Sermon K, Lissens W, Vandervorst M, Vanderfaeillie A, De Rijcke M, De Vos A, Henderix P, Van De Velde H, Van Steirteghem A, Liebaers I: Clinical application of preimplantation genetic diagnosis for cystic fibrosis. Prenat Diagn 2000, 20:571–581
107. Blaszczyk A, Tang YX, Dietz HC, Adler A, Berkeley AS, Krey LC,

Grifo JA: Preimplantation genetic diagnosis of Marfan's syndrome. J Assist Reprod Genet 1998, 15:281–284
108. Dreesen JC, Bras M, de Die-Smulders C, Dumoulin JC, Cobben JM, Evers JL, Smeets HJ, Geraedts JP: Preimplantation genetic diagnosis of spinal muscular atrophy. Mol Hum Reprod 1998, 4:881–885
109. Fallon L, Harton GL, Sisson ME, Rodriguez E, Field LK, Fugger EF, Geltinger M, Sun Y, Dorfmann A, Schoener C, Bick D, Schulman J, Levinson G, Black SH: Preimplantation genetic diagnosis for spinal muscular atrophy type I. Neurology 1999, 53:1087–1090
110. Daniels G, Pettigrew R, Thornhill A, Abbs S, Lashwood A, O'Mahoney F, Mathew C, Handyside A, Braude P: Six unaffected livebirths following preimplantation diagnosis for spinal muscular atrophy. Mol Hum Reprod 2001, 7:995–1000
111. Gibbons WE, Gitlin SA, Lanzendorf SE, Kaufmann RA, Slotnick RN, Hodgen GD: Preimplantation genetic diagnosis for Tay-Sachs disease: successful pregnancy after pre-embryo biopsy and gene amplification by polymerase chain reaction. Fertil Steril 1995, 63: 723–728
112. Orita M, Suzuki Y, Sekiya T, Hayashi K: Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. Genomics 1989, 5:874–879
113. El-Hashemite N, Wells D, Delhanty JD: Single cell detection of beta-thalassaemia mutations using silver stained SSCP analysis: an application for preimplantation diagnosis. Mol Hum Reprod 1997, 3:693–698
114. Sheffield VC, Cox DR, Lerman LS, Myers RM: Attachment of a 40-base-pair G + C-rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. Proc Natl Acad Sci USA 1989, 86:232–236
115. Kanavakis E, Vrettou C, Palmer G, Tzetis M, Mastrominas M, Traeger-Synodinos J: Preimplantation genetic diagnosis in 10 couples at risk for transmitting beta-thalassaemia major: clinical experience including the initiation of six singleton pregnancies. Prenat Diagn 1999, 19:1217–1222
116. Holding C, Bentley D, Roberts R, Bobrow M, Mathew C: Development and validation of laboratory procedures for preimplantation diagnosis of Duchenne muscular dystrophy. J Med Genet 1993, 30:903–909
117. Pickering SJ, McConnell JM, Johnson MH, Braude PR: Use of a polymorphic dinucleotide repeat sequence to detect non-blastomeric contamination of the polymerase chain reaction in biopsy samples for preimplantation diagnosis. Hum Reprod 1994, 9:1539–1545
118. Walsh PS, Fildes NJ, Reynolds R: Sequence analysis and characterization of stutter products at the tetranucleotide repeat locus vWA. Nucleic Acids Res 1996, 24:2807–2812
119. Harton GL, Tsipouras P, Sisson ME, Starr KM, Mahoney BS, Fugger EF, Schulman JD, Kilpatrick MW, Levinson G, Black SH: Preimplantation genetic testing for Marfan syndrome. Mol Hum Reprod 1996, 2:713–715
120. Apessos A, Abou-Sleiman PM, Harper JC, Delhanty JD: Preimplantation genetic diagnosis of the fragile X syndrome by use of linked polymorphic markers. Prenat Diagn 2001, 21:504–511
121. Sermon K, Lissens W, Messiaen L, Bonduelle M, Vandervorst M, Van Steirteghem A, Liebaers I: Preimplantation genetic diagnosis of Marfan syndrome with the use of fluorescent polymerase chain reaction and the automated laser fluorescence DNA sequencer. Fertil Steril 1999, 71:163–166
122. Dreesen JC, Jacobs LJ, Bras M, Herbergs J, Dumoulin JC, Geraedts JP, Evers JL, Smeets HJ: Multiplex PCR of polymorphic markers flanking the CFTR gene: a general approach for preimplantation genetic diagnosis of cystic fibrosis. Mol Hum Reprod 2000, 6:391–396
123. Eftedal I, Schwartz M, Bendtsen H, Andersen AN, Ziebe S: Single intragenic microsatellite preimplantation genetic diagnosis for cystic fibrosis provides positive allele identification of all CFTR genotypes for informative couples. Mol Hum Reprod 2001, 7:307–312
124. Braude PR, De Wert GM, Evers-Kiebooms G, Pettigrew RA, Geraedts JP: Non-disclosure preimplantation genetic diagnosis for Huntington's disease: practical and ethical dilemmas. Prenat Diagn 1998, 18:1422–1426
125. Lee SH, Kwak IP, Cha KE, Park SE, Kim NK, Cha KY: Preimplantation diagnosis of non-deletion Duchenne muscular dystrophy (DMD) by linkage polymerase chain reaction analysis. Mol Hum Reprod 1988, 4:345–349
126. Sutterlin M, Sleiman PA, Onadim Z, Delhanty J: Single cell detection of inherited retinoblastoma predisposition. Prenat Diagn 1999, 19: 1231–1236
127. Sermon K, Lissens W, Joris H, Seneca S, Desmyttere S, Devroey P, Van Steirteghem A, Liebaers I: Clinical application of preimplantation diagnosis for myotonic dystrophy. Prenat Diagn 1997, 17:925–932
128. Sermon K, Seneca S, Vanderfaeillie A, Lissens W, Joris H, Vandervorst M, Van Steirteghem A, Liebaers I: Preimplantation diagnosis for fragile X syndrome based on the detection of the non-expanded paternal and maternal CGG. Prenat Diagn 1999, 19:1223–1230
129. Dreesen JC, Geraedts JP, Dumoulin JC, Evers JL, Pieters MH: RS46(DXS548) genotyping of reproductive cells: approaching preimplantation testing of the fragile-X syndrome. Hum Genet 1995, 96:323–329
130. Piyamongkol W, Harper JC, Sherlock JK, Doshi A, Serhal PF, Delhanty JD, Wells D: A successful strategy for preimplantation genetic diagnosis of myotonic dystrophy using multiplex fluorescent PCR. Prenat Diagn 2001, 21:223–232
131. Thornhill AR, Monk M: Cell recycling of a single human cell for preimplantation diagnosis of X-linked disease and dual sex determination. Mol Hum Reprod 1996, 2:285–289
132. Pettigrew R, Kuo HC, Scriven P, Rowell P, Pal K, Handyside A, Braude P, Ogilvie CM: A pregnancy following PGD for X-linked autosomal dominant incontinentia pigmenti (Bloch-Sulzberger syndrome): case report. Hum Reprod 2000, 15:2650–2652
133. Rechitsky S, Freidine M, Verlinsky Y, Strom CM: Allele dropout in sequential PCR and FISH analysis of single cells (cell recycling). J Assist Reprod Genet 1996, 13:115–124
134. Pals G, Young C, Mao HS, Worsham MJ: Detection of a single base substitution in a single cell using the LightCycler. J Biochem Biophys Methods 2001, 47:121–129
135. Underhill PA, Jin L, Lin AA, Mehdi SQ, Jenkins T, Vollrath D, Davis RW, Cavalli-Sforza LL, Oefner PJ: Detection of numerous Y chromosome biallelic polymorphisms by denaturing high-performance liquid chromatography. Genome Res 1997, 7:996–1005
136. Drury KC, Liu MC, Lilleberg S, Kipersztok S, Williams RS: Results on single cell PCR for Huntington's gene and WAVE product analysis for preimplantation genetic diagnosis. Mol Cell Endocrinol 2001, 183 (Suppl 1): S1–S4
137. Hughes TR, Roberts CJ, Dai H, Jones AR, Meyer MR, Slade D, Burchard J, Dow S, Ward TR, Kidd MJ, Friend SH, Marton MJ: Widespread aneuploidy revealed by DNA microarray expression profiling. Nat Genet 2000, 25:333–337
138. Harper JC, Wells D: Recent advances and future developments in PGD. Prenat Diagn 1999, 19:1193–1199
139. Burkardt HJ: Standardization and quality control of PCR analyses. Clin Chem Lab Med 2000, 38:87–91
140. Simoni M: Molecular diagnosis of Y chromosome microdeletions in Europe: state-of-the-art and quality control. Hum Reprod 2001, 16: 402–409
141. Verlinsky Y, Munne S, Simpson JL, Kuliev A, Ao A, Ray P, Sermon K, Martin R, Strom C, Van Steirteghem A, Veiga A, Drury K, Williams S, Ginsberg N, Wilton L: Current status of preimplantation diagnosis. J Assist Reprod Genet 1997, 14:72–75
142. Viville S, Pergament D: Results of a survey of the legal status and attitudes towards preimplantation genetic diagnosis conducted in 13 different countries. Prenat Diagn 1998, 18:1374–1380
143. Fasouliotis SJ, Schenker JG: Preimplantation genetic diagnosis principles and ethics. Hum Reprod 1998, 13:2238–2245
144. Beyleveld D: Is embryo research and preimplantation genetic diagnosis ethical? Forensic Sci Int 2000, 113:461–475
145. Draper H, Chadwick R: Beware! Preimplantation genetic diagnosis may solve some old problems but it also raises new ones. J Med Ethics 1999, 25:114–120
146. King DS: Preimplantation genetic diagnosis and the "new" eugenics. J Med Ethics 1999, 25:176–182
147. American Society for Reproductive Medicine and Society for Assisted Reproductive Technology Practice Committee Report: Preimplantation Genetic Diagnosis. June, 2001

EXHIBIT 5

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Multiplex PCR of polymorphic markers flanking the *CFTR* gene; a general approach for preimplantation genetic diagnosis of cystic fibrosis*

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Cystic fibrosis (CF) is the first monogenic disorder for which single cell preimplantation genetic diagnosis (PGD) has been successfully applied. The spectrum of mutations in CF is extremely heterogeneous, and hence, the development of mutation-specific PGD protocols is impracticable. The current study reports the development and evaluation of a general multiplex marker polymerase chain reaction (PCR) protocol for PGD of CF. Four closely linked highly polymorphic $(CA)_n$ repeat markers D7S523, D7S486, D7S480 and D7S490, flanking the cystic fibrosis transmembrane regulator (*CFTR*) gene, were used. In 99% of the single cells tested (100 leukocytes and 50 blastomeres), multiplex PCR results were obtained and the overall allelic drop out (ADO) rate varied from 2 to 5%. After validation for the presence of ADO and additional alleles, 95% of the multiplex PCR results were accepted to construct the marker genotypes. Depending on the genotype of the couple, and taking into account the embryos lost for transfer due to validation criteria (5%), ADO (0–2%) and single recombination (1.1–3%), in general >90% of the embryos could be reliably genotyped by PGD using a single blastomere. The risk of misdiagnosis equals the chance of a double recombination between informative flanking markers and is <0.05%. Therefore, this polymorphic and multi-allelic marker system is a reliable and generally applicable alternative for mutation-directed PGD protocols. Furthermore, it provides a test for the origin of the detected genotype and also gives an indication of the chromosomal ploidy status of the blastomere tested.

Key words: cystic fibrosis/multiplex marker PCR/preimplantation genetic diagnosis/single cell diagnosis

Introduction

Preimplantation genetic diagnosis (PGD) is a combination of IVF and the genetic diagnosis of embryos at the early cleavage stage. It allows the selection and transfer of unaffected preimplantation stage embryos to the uterus. For couples at risk of transferring a genetic disorder to their offspring, PGD offers an alternative to prenatal diagnosis. By choosing PGD, the difficult decision of pregnancy termination after genetic diagnosis by chorionic villus sampling or amniocentesis in the first and second trimesters of gestation can be avoided. In 1990, the first successful PGD was reported for a number of X-linked genetic disorders by sexing embryos using Y-specific DNA polymerase chain reaction (PCR) amplification (Handyside *et al.*, 1990). The first single gene defect for which PGD was successfully applied, was the triplet basepair Δ*F508* deletion in the cystic fibrosis transmembrane regulator (*CFTR*) gene, also by means of PCR (Handyside *et al.*, 1992). Since then the number of disorders for which PGD can be performed is growing slowly, but steadily. Technically, PGD remains a challenge as only one or two blastomeres are available for analysis and this has to be completed within one day. The genetic analysis on one single blastomere has to meet high standards of PCR efficiency, allelic drop out (ADO) rate, reliability, and contamination control (Lissens *et al.* 1996). Therefore a PGD protocol is put through an extensive preclinical trial before it can be introduced into a clinic. In most PGD clinics, the cystic fibrosis (CF) Δ*F508* deletion is one of the single gene defects for which PGD is offered as an alternative for prenatal diagnosis.

CF is a common autosomal recessive genetic disorder with a prevalence of ~1 in 2500 live births and a carrier frequency of ~1 in 25 in the North Western European population (Findlay, 1997; Tsui and Durie, 1997). The disease phenotype is heterogeneous and depends on the specific mutation in the *CFTR* gene. Clinical characteristics of CF are accumulations of dehydrated mucus, resulting in chronic obstructive respiratory disease, pancreatic enzyme deficiency and obstruction of the small intestine. Also congenital bilateral absence of the vas deferens (CBAVD) is present in ~95% of the male CF patients, leading to male infertility (Chillón *et al.*, 1995). Without treatment the disease usually causes early death from pulmon-

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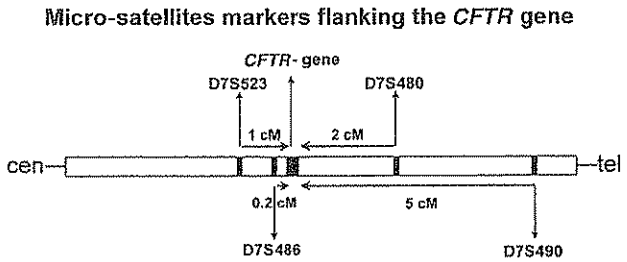

Figure 1. Micro-satellite markers flanking the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. The positions of the dinucleotide repeat markers relative to the *CFTR* gene are depicted. Genetic distances are given in centi-Morgan (cM). Cen = centromere; tel = telomere.

ary infections. CF has a considerable impact on the quality of life and, although improved medical care often preserves life into adulthood, the median life expectancy is only 30 years (Tsui and Durie, 1997). Worldwide more than 700, mostly rare, CF-related mutations have been identified (Tsui and Durie, 1997). In the Netherlands, a phenylalanine deletion at amino acid position 508 (*ΔF508*) is the most common mutation with a frequency of 77% of the CF chromosomes (Haigh and Kazazian, 1994). In 60% of CF couples, both partners carry the *ΔF508* mutation and a PGD protocol for this specific mutation is operational in our clinic (Liu *et al.*, 1992). However, for the remaining 40% in which other CF mutations are involved no such test is available yet. Because the development of mutation-specific PGD protocols for all mutations other than the homozygous *ΔF508* mutation is impracticable, we developed a general marker-based protocol for PGD of CF.

Four closely linked highly polymorphic $(CA)_n$ repeat markers flanking the *CFTR* gene were used; D7S523 and D7S486 with heterozygote frequencies of 80 and 81%, located 1 and 0.2 cM proximal to the *CFTR* gene, and D7S480 and D7S490 with heterozygote frequencies of 86 and 78%, located 2 and 5 cM distal to the gene (Figure 1; de Vries *et al.*, 1996). This protocol can extend the number of CF couples to whom PGD can be offered by nearly 2/3 of all the patients that request PGD for the CF *ΔF508* mutation. To perform a reliable marker-based CF diagnosis family studies are required to determine the wild-type and CF risk haplotype. At least two flanking markers must be informative. The aim of this study was to investigate the feasibility of a general PGD protocol using a multiplex PCR of the four markers at the single cell level.

Materials and methods

Heterozygote frequencies of micro-satellite markers

To ascertain the heterozygote frequencies in the Dutch population of the $(CA)_n$ repeat markers, multiplex PCR was performed on 300 ng genomic DNA as described below, with a reduced number of 35 amplification cycles. DNA was obtained from 29 unrelated individuals of the Dutch population, and isolated from peripheral blood leukocytes according to standard protocols (Miller *et al.*, 1988). The observed and published (de Vries *et al.*, 1996) heterozygote frequency data were analysed using the χ^2 test or Fisher's exact test where appropriate.

Collection of human leukocytes and blastomeres

Human leukocytes and blastomeres were used as single cells for testing the PCR method. Single leukocytes were collected in 2 µl Ca^{2+} and Mg^{2+}-free phosphate-buffered saline solution (PBS) with 1% polyvinylpyrrolidone (PVP) molecular weight 360 kDa (Sigma, Aldrich Chemie BV, Zwijdrecht, The Netherlands) and 0.1 mg/ml Phenol Red (Sigma), with the help of a micromanipulator (ONO-121; Narishige, Paes Nederland BV) mounted on an inverted microscope (IX-70; Olympus, Zoeterwoude, The Netherlands). After transferring the cells to a 0.2 ml reaction tube, cellular DNase heat inactivation was accomplished by a 10 min incubation at 65°C. Cells were stored at –20°C until PCR was performed. Blastomeres were obtained from human embryos that were donated after IVF treatment. They were considered to be unsuitable for freezing. Seven surplus embryos after IVF were used from three different couples. Blastomeres were collected from these embryos after removing the zona pellucida by 3–5 min incubation in a 1/1 mixture of 500 IU/ml pronase (Sigma) and PBS. The blastomeres were separated from each other by gently flushing in a small pipette. Subsequently, they were rinsed in three droplets of PBS, with 1% PVP and Phenol Red (0.1 mg/ml), and transferred into a 0.2 ml reaction tube with the help of a dissection microscope. To obtain information on the maternal and paternal marker genotypes of the blastomeres, cumulus cells and spermatozoa served as PCR target material respectively. Cellular DNase inactivation and storage of the collected cells was performed as described previously. Blastomeres were only collected after couples had given consent that surplus embryos were used for these experiments. The protocol was approved by the local Ethical Committee.

PCR procedure

Prior to multiplex PCR, the alkaline lysis buffer and the PCR mix without primers or *Taq/Pwo* DNA polymerase (Roche Diagnostics, Nederland BV, Almere, The Netherlands), were decontaminated from DNA by UV-C irradiation for 1 h using an UV-C lamp type TUV 30W/G30T8 longlife (Philips, Eindhoven, The Netherlands). Blank samples were included in every PCR series to monitor DNA contamination. Cells were lysed by adding 2.5 µl of alkaline lysis buffer [50 mmol/l dithiothreitol (DTT; Pharmacia Biotech, Benelux, Roosendaal, The Netherlands)/200 mmol/l NaOH] followed by 10 min of incubation at 65°C. After the cell lysis, multiplex PCR was performed with the GeneAmp® PCR System 9700 (Perkin-Elmer Applied Biosystems, Nieuwerkerk a/d, Ijssel, The Netherlands) using the Expand™ Long Template PCR System (Roche Diagnostics). The PCR reaction was performed in a total volume of 25 µl and contained 1× Buffer® and 2.5 IU of *Taq/Pwo* polymerase provided by the manufacturer (Roche Diagnostics), 0.2 mmol/l dNTP from each of the four deoxynucleotide triphosphates (dGTP/dATP/dCTP/dTTP; Pharmacia), 20 mmol/l Tricine pH 4.95 (Merck, Nederland BV, Amsterdam, The Netherlands) to neutralize the alkaline lysis buffer and the primersets of which the forward primer is fluorescently labelled (3 pmol 490R (AGC.TA-C.TTG.CAG.TGT.AAC.AGC.ATT.T)/490F-TET (CCT.TGG.GCC.AAT.AAG.GTA.AG), 10 pmol 486R (GCC.C-AG.GTG.ATT.GAT.A-GT.GC)/486F-HEX(AAA.GGC.CAA.TGG.T-AT.ATC.CC), 10 pmol 480R (AGC.TAC.CAT.AGG.GCT.GGA.GG)/480F-HEX(CTT.GGG.GAC.TGA.ACC.ATC. TT) and 20 pmol 523R (AAA.ACA.TTT.CC-A.TTA.CCA.CTG)/523F-HEX(CTG.ATT.CA-T.AGC.AGC.ACT.TG) (Gyapay *et al.*, 1994). PCR was started with an initial 5 min denaturation step at 95°C followed by 55 cycles of 30 s denaturation at 95°C, 60 s annealing at 55°C and 60 s elongation at 68°C with a final elongation step of 5 min at 68°C. PCR products were separated on a 8% Long Ranger (FMC BioProducts, Sanver TECH, Heerhugowaard, The Netherlands) denaturing polyacrylamide gel mounted on a ABI Prism 377 DNA Sequencer with automated

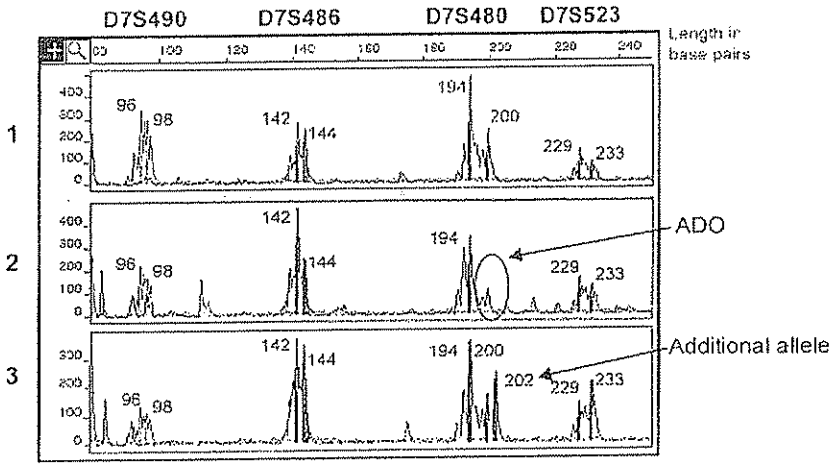

Figure 2. Electropherogram obtained after GeneScan analysis of micro-satellite marker multiplex polymerase chain reaction products separated on 8% denaturing polyacrylamide gel. On top of the electropherogram the marker name is put above the corresponding marker alleles (peaks). The length of the marker alleles (in bp), is depicted on top of the electropherogram, and next to the corresponding marker alleles.

fluorescent scanning detection and analysed using GeneScan Analysis Software version 2.1, to size the PCR fragments (PE Applied Biosystems). Within 10 h after sample preparation, the entire procedure of cell lysis, PCR amplification and PCR product analysis was completed.

Results

Heterozygote frequencies of micro-satellite markers

Heterozygote frequencies of the markers used were determined by genotyping 29 unrelated individuals from the Dutch population. The heterozygote frequencies of D7S523, D7S486, D7S480 and D7S490 were 81, 77, 87 and 80% respectively.

Optimizing single cell multiplex PCR

Initially 50 pg amounts of genomic DNA were used as target material, succeeded by single cells (leukocytes or blastomeres) in a later stage. The multiplex PCR of $(CA)_n$ repeat markers was optimized by looking for optimal but mutual PCR conditions of the individual markers, followed by combining the primer sets in one PCR reaction. However, combination of the primer sets in one PCR reaction required careful adjustment of the primer concentrations to obtain marker signals that could be scored within the same detection range. Furthermore, marker D7S490 is labelled with a different fluorescent label to distinguish it from background peaks from upstream markers. The electropherograms in Figure 2 show the single cell multiplex PCR results of blastomeres with the same marker genotypes, heterozygous for the four markers used. Electropherogram 1 demonstrates the complete genotype whereas in electropherogram 2 (ADO) and in electropherogram 3 the presence of an additional allele can be observed. Additional alleles are defined as alleles that cannot be deduced from the parents and are of non-paternal or maternal origin, either caused by contamination or PCR artefacts.

Single cell testing

The optimized multiplex marker PCR was evaluated for PCR efficiency, ADO rate and the frequency of additional alleles. A total of 100 single leukocytes isolated from fresh blood obtained from four unrelated individuals, and 50 nucleated single blastomeres, obtained from seven surplus embryos from three couples, were analysed by multiplex PCR. All 100 leukocytes revealed positive PCR signals for each of the four markers tested whereas ADO rates determined in cells heterozygote for the concerning markers range from 1% for D7S490 to 6% for D7S480 as shown in Table I. Of the 50 blastomeres, 49 gave positive PCR signals for each of the four markers tested and ADO rates varied from 0% for D7S523 to 7% for D7S486. The overall PCR efficiency of single cells tested was 99%, and the overall ADO rate varied from 2 to 5% (Table I). All 25 blanks included in the single cell test series were negative.

The multiplex PCR results of the amplified leukocytes and blastomeres were validated with respect to the occurrence of ADO and additional alleles before they were accepted to construct the marker genotypes (Table II). Multiplex PCR results were considered to be acceptable for marker genotype construction, when all markers gave amplification results and no more than one ADO event and/or one additional allele were detected. Although these criteria are somewhat arbitrary, the occurrence of ADO and the presence of additional alleles are an indication that the PCR results may not be reliable. No significant differences were found between leukocytes and blastomeres. The complete genotype without additional alleles or ADO was obtained in 73% of 149 single cells with a positive PCR result. In 22% of the tested single cells one additional allele and/or one ADO event was detected. In only 5% of the cases, more than one additional allele and/or more than one ADO event occurred.

Furthermore, we demonstrated the inheritance of paternal and maternal marker haplotypes in 46 of 49 blastomeres which were accepted for genotyping according to the validation criteria. Paternal and maternal marker haplotypes were determined from the amplification of spermatozoa and cumulus cells respectively. Figure 3 shows the electropherograms obtained from the cumulus cells and sperm cells of one couple,

Table I. Single cell multiplex polymerase chain reaction (PCR) of the micro-satellite markers flanking the *CFTR* gene: PCR efficiency and allelic drop-out (ADO)

Marker	PCR efficiency			ADO		
	Leukocytes (%)	Blastomeres (%)	total (%)	Leukocytes (%)	Blastomeres (%)	total (%)
D7S523	100	98	99	3	0	2
D7S486	100	98	99	2	7	3
D7S480	100	98	99	6	3	5
D7S490	100	98	99	1	4	2
	n = 100	n = 50	n = 150	n =72–100[a]	n = 30–46[a]	n = 102–140[a]

No significant differences were noted between leukocytes and blastomeres. Data were analysed using the χ^2 test or Fisher's exact test where appropriate.
[a]The n values of the cells used to determine the ADO rate vary, because not all single cells are heterozygous for the concerning markers.

Table II. Validation of obtained multiplex polymerase chain reaction (PCR) results

Multiplex PCR profile	Leukocytes (%)	Blastomeres (%)	Total (%)
No additional allele and/or no allele ADO	73	74	73
One additional allele and/or one allele ADO	22	20	22
>one additional allele and/or >one allele ADO	5	6	5
	n = 100	n = 49	n = 149

No significant differences were noted between leukocytes and blastomeres. Data were analysed using the χ^2 test or Fisher's exact test where appropriate.

electropherogram 1 and 2 respectively. Also the electropherograms (3, 4 and 5) of three single blastomeres collected from three different surplus embryos from this couple after IVF are shown. Blastomeres represented by electropherogram 3, 4 and 5 clearly demonstrated maternal and paternal alleles for all the four markers tested. In electropherogram 4, all markers revealed two different alleles demonstrating that this embryo was heterozygous for all the markers tested. The embryo represented by the blastomere analysed in electropherogram 3 was heterozygous for the markers D7S490 and D7S523 and homozygous for D7S486 and D7S480. Electropherogram 5 shows three different marker alleles (two maternal and one paternal allele) for the markers D7S490, D7S486 and D7S480. Marker D7S523 reveals only two different alleles. However it is likely that this embryo inherited both maternal 233 bp *D7S523* alleles and that it was trisomic for the four markers amplified.

Discussion

The heterozygote frequencies of the $(CA)_n$ repeat markers used in our population did not differ significantly from the heterozygote frequencies published by de Vries *et al.* (1996). Based on these heterozygote frequencies, we conclude that in ~87% of the couples at least one marker on each side of the *CFTR* gene is informative, which is a precondition for a reliable marker-based CF diagnosis. Although the confidence interval of this estimate is large, the vast majority of couples will be informative for this marker-based approach for PGD of CF. In single cell multiplex PCR, PCR efficiency, validation criteria, ADO and recombination events define the number of cells for which a reliable marker genotype can be constructed, and the number of embryos for which a marker-based CF diagnosis can be performed. Consequently these factors affect the number of embryos suitable for transfer in case of a PGD.

The PCR efficiency and the validation criteria affect the number of single cells for which a reliable genotype can be constructed, irrespective of the CF genotype of the single cells tested. In 99% of the single cells amplified, multiplex marker PCR data were obtained. After validating these data according to the criteria stated previously, we could reliably genotype 95% of the single cells for the markers used. This suggests that ~5% of the blastomeres obtained in a PGD cycle would consequently result in an embryo lost for transfer.

In the single cells for which a marker genotype could be constructed (95%), a fraction will result in embryos lost for transfer due to ADO and recombination events. The effect of ADO and single recombination events depend on the CF genotype. In cells containing both the maternal and paternal wild-type or CF-risk marker haplotypes, ADO or a single recombination event does not lead to embryos lost for transfer, or the transfer of an affected embryo. In these homozygous wild-type or CF-mutated cells one marker haplotype cannot be constructed, but the CF status can be ascertained by the other marker haplotype. However in carriers with two different marker haplotypes, the wild-type and CF risk haplotype, ADO and recombination events can prevent identification of the healthy marker haplotype. In case of PGD, these embryos are lost for transfer because only the risk haplotype can be constructed. The exact contribution of ADO and recombination events has to be derived from the parental genotypes prior to PGD and is predetermined by which markers and the number of markers that are informative. A thorough family study prior to PGD is essential to reliably construct the wild-type and CF risk marker haplotypes. If this is not the case, a single recombination event can lead to 50% of the embryos lost for transfer.

In the optimal situation where all four markers are informative, ADO does not result in carrier embryos lost for transfer.

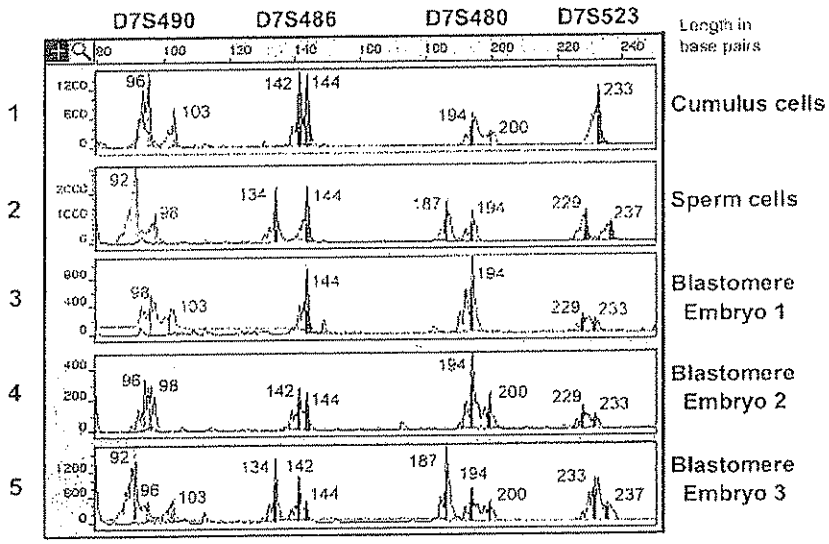

Figure 3. Electropherogram of multiplex polymerase chain reaction of the four micro-satellites marker performed on cumulus cells, sperm cells and single blastomeres obtained from surplus embryos after IVF.

After ADO (Table I) of one marker allele of the healthy haplotype, there are still markers left flanking the *CFTR* gene, making construction of the healthy marker haplotype possible. However, if three markers are informative, the chance that a CF carrier embryo cannot be transferred due to ADO, is determined by half the ADO rate of the sole distal or proximal marker. In the ADO affected carrier embryos, the incidence of the risk haplotype being lost is equal to that of the healthy wild-type marker haplotype being lost. Therefore, the chance that only the risk haplotype is ascertained is 1–2.5%. If only two markers are informative, one on each site of the *CFTR* gene, this chance is determined by the sum of half the ADO rates of the two flanking informative markers and varies from 2 to 4%. Because carrier embryos are 50% of the total embryo population, the overall contribution of ADO to embryos lost for transfer varies from 0 to 2%, depending on the number and type of informative markers.

Recombination occurs in 2.2–6% of the paternal and maternal haplotypes depending on which flanking markers are informative. Therefore the total change on recombination varies from 4.4 to 12%. However, recombination events, similar to ADO events, only lead to embryos being lost for transfer in carrier embryos. Of the heterozygous embryos affected by recombination 50% still can be transferred because the wild-type marker haplotype can be ascertained. Moreover, of the total embryo population, 50% are CF carriers. This means that due to recombination events, and depending on the genetic distances of the informative markers, 1.1–3% of the embryos are lost for transfer.

ADO and single recombination events do not result in erroneous transfer of an affected embryo provided that the marker haplotype unaffected by ADO and recombination can be reliably ascertained. On the contrary, double recombination between flanking heterozygous markers of the paternal or maternal CF risk marker haplotype may lead to a marker-based CF misdiagnosis in a CF compound heterozygous or homozygous embryo. The double recombination event of such an embryo can result in the detection of a wild-type marker haplotype although the CF mutation is present. Hence the embryo displays a marker genotype of a carrier embryo although it is compound heterozygous or homozygous for the CF mutation. However, the risk of transferring an affected embryo with a double recombination between flanking markers is $<0.05\%$.

An additional source of misdiagnosis can be caused by contamination with foreign DNA. Precautions taken to avoid contamination as described in Materials and methods appear to be sufficient. This can be concluded from the 25 blank samples included in the single cell test series to monitor contamination, which were all negative. Furthermore this multiplex PCR marker system provides a test for contamination. Prior to PGD the maternal and paternal marker haplotypes are determined and the expected marker alleles for testing a single cell are known. Therefore the use of this highly polymorphic multiplex PCR system provides also a control for contamination. The four dinucleotide repeat marker alleles identify the origin of the amplified DNA. They confirm the amplification of blastomeric template DNA or expose contamination by foreign DNA as has been shown previously (Pickering *et al.*, 1994; Findlay *et al.*, 1995). Pickering used a dinucleotide repeat sequence to obtain a crude DNA fingerprint whereas Findlay used six tetranucleotide micro-satellite sequences to determine a DNA fingerprint from single cells. Deduced from the allele frequencies of the individual four markers used (data not shown), the observed genotype has a <1 in 24 000 chance of not being from the amplified cell.

An additional advantage of this highly polymorphic multi allelic marker PCR system is demonstrated in Figure 3. Although the markers only give information about the number of chromosomes 7, e.g. trisomy 7 (Figure 3; embryo 3), an indication about the ploidy status of the tested embryos is obtained as well. This prevents triploid embryos from being

transferred. The use of fluorescent PCR of polymorphic small tandem repeats for determining chromosomal trisomies have been reported for application in prenatal diagnosis and PGD (Findlay *et al.*, 1998; Verma *et al.*, 1998; Blake *et al.*, 1999).

Conclusions

Multiplex PCR is a reliable and generally applicable alternative for mutation-directed PGD protocols, irrespective of the CF mutations involved. The protocol expands the number of CF couples to whom PGD can be offered, by nearly 2/3 of all the patients that request PGD for the CF *ΔF508* mutation. It makes the development of individual mutation-directed PGD protocols redundant, provided that the couple at risk is informative for the markers used. However, the multiplex marker-based CF diagnosis requires a thorough family study to ascertain the CF risk and wild-type marker haplotype. The exact number of family members to be analysed to construct haplotypes depends on the family constitution. The majority of couples who are carriers of a recessive genetic disorder have affected offspring, limiting the number of family members to be analysed for the markers used, to four: the couple at risk; their affected child; and an unaffected sibling. If no unaffected brothers or sisters are available, paternal and maternal grandparents of the index patient may be analysed to rule out recombination, and accurately determine the CF risk and wild-type marker haplotype. Additional information on the paternal haplotypes can be obtained by analysing single sperm cells. This family study is not necessary for mutation-directed protocol for which only information about the mutations involved is required. Nevertheless, for single cell analyses the marker-based approach clearly is advantageous over the direct mutation approach. Unlike the marker-based CF diagnosis, ADO in mutation-directed protocols can lead to the transfer of affected embryos in PGD of compound heterozygotes. If (due to ADO) one mutant allele is not detected and only the wild-type allele is observed, the embryo can be genotyped as a carrier of the only detected mutation. Hence transfer of an affected embryo can occur. Furthermore, the polymorphic and multi-allelic character of this marker system provides a test for the origin of the detected genotype and gives an indication about the chromosomal ploidy of the blastomere tested. This is not possible with the mutation-directed protocols, in which information is obtained of the mutant and wild-type alleles only. Depending on the genotype of the couple, and taking into account the embryos lost for transfer by the validation criteria (5%), the ADO event (0–2%) and the single recombination event (1.1–3%), >90% of the embryos can be reliably genotyped on a single blastomere. Moreover, the risk of misdiagnosis equals the chance of a double recombination between flanking markers and is <0.05%. Therefore this approach will enable PGD as a choice for virtually all CF carriers provided that at least two flanking markers are informative.

References

Blake, D., Tan, S.L. and Ao, A. (1999) Assessment of multiplex fluorescent PCR for screening single cells for trisomy 21 and single gene defects. *Mol. Hum. Reprod.*, 5, 1166–1175.

Chillón, M., Casals, T., Mercier, B. *et al.* (1995) Mutations in the cystic fibrosis gene and absence of the vas deferens. *N. Engl J. Med.*, **332**, 1475–1480.

Findlay, I., Urquhart, A., Quirke, P. *et al.* (1995) Simultaneous DNA 'fingerprinting', diagnosis of sex and single-gene defect status from single cells. *Mol. Hum. Reprod.*, **1**, see *Hum. Reprod.*, **10**, 1005–1013.

Findlay, I. (1997) Approaches for cystic fibrosis diagnosis. *Hum. Reprod.*, **12** (Natl. Suppl.), *JBFS*, 2, 166–169.

Findlay, I., Tóth, T., Matthews, P. *et al.* (1998) Rapid trisomy diagnosis (21, 18, and 13) using fluorescent pcr and short tandem repeats: applications for prenatal diagnosis and preimplantation genetic diagnosis. *J. Assist. Reprod. Genet.*, **15**, 266–275.

Gyapay, G., Morisette, J., Vignal, A. *et al.* (1994) The 1993–94 Généthon human genetic linkage map. *Nature Genet.*, 7, 246–339.

Haigh, H. and Kazazian, Jr. (1994) Population variation of common cystic fibrosis mutations. *Hum. Mutat.*, 4, 167–177.

Handyside, A.H., Kontogianne, E.H., Hardy, K. *et al* (1990) Pregnancies from biopsied human preimplantation embryos sexed by Y-specific DNA amplification. *Nature*, **344**, 768–770.

Handyside, A.H., Lesko, J.G., Tarín, J.J. *et al.* (1992) Birth of a normal girl after *in vitro* fertilisation and preimplantation diagnostic testing for cystic fibrosis. *N. Engl J. Med.*, **327**, 905–909.

Lissens, W., Sermon K., Staessen, C. *et al.* (1996) Review: Preimplantation diagnosis of inherited disease. *J. Inherit. Metab. Dis.*, **19**, 709–723.

Liu, J., Lissens, W., Devroey P. *et al.* (1992) Efficiency and accuracy of polymerase-chain-reaction assay for cystic fibrosis allele ΔF508 in single cell. *Lancet*, **339**, 1190–1192.

Miller, S.A., Dykes, D.D. and Polesky, H.F. (1988) A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res.*, **16**, 1215.

Pickering, S.J., McConnell J.M., Johnson M.H. *et al.* (1994) Use of a polymorphic dinucleotide repeat sequence to detect non-blastomeric contamination of the polymerase chain reaction in biopsy samples for preimplantation diagnosis. *Hum. Reprod.*, **9**, 1539–1545.

Tsui, L. and Durie P. (1997) Genotype and phenotype in cystic fibrosis. *Hospital Practice*, 115–142.

Verma, L., Macdonald, F., Leedham, P. *et al.* (1998) Rapid and simple prenatal DNA diagnosis of Down's syndrome. *Lancet*, **352**, 9–12.

de Vries, H.G., van der Meulen, M.A., Rozen, R. *et al.* (1996) Haplotype identity between individuals who share a *CFTR* mutation allele 'identical by descent': demonstration of the usefulness of the haplotype-sharing concept for gene mapping in real populations. *Hum. Genet.*, **98**, 304–309.

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